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The Journal of Clinical Investigation

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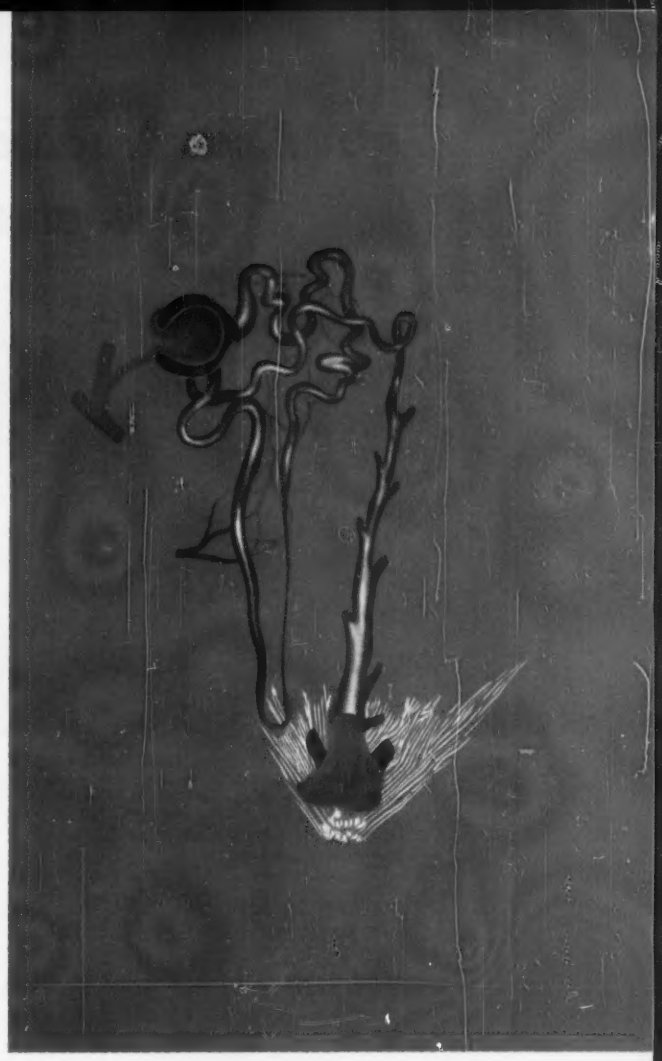

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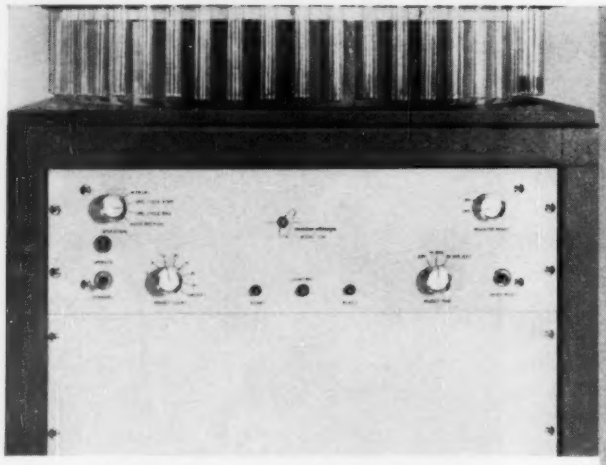

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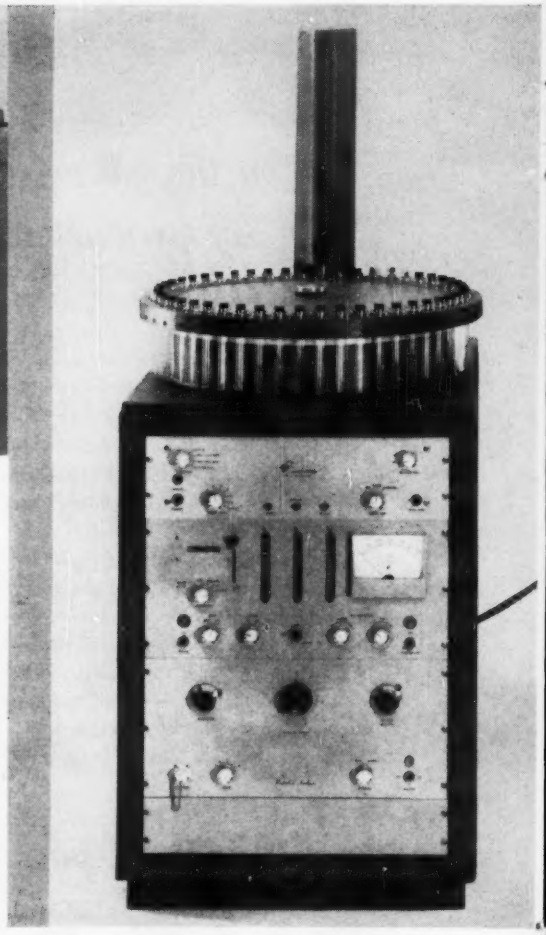

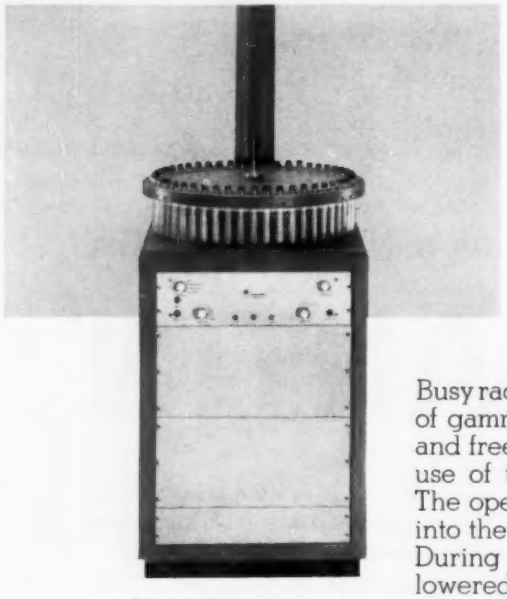

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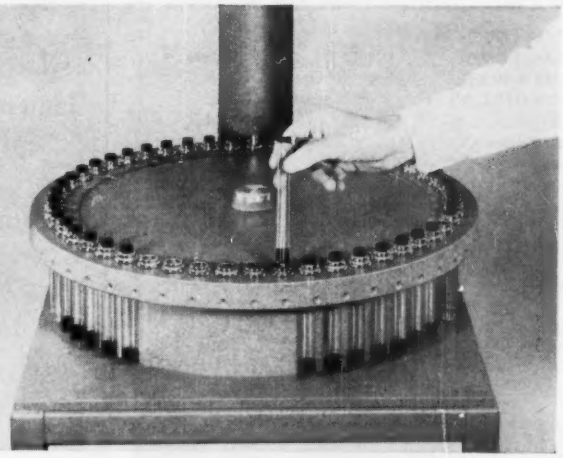

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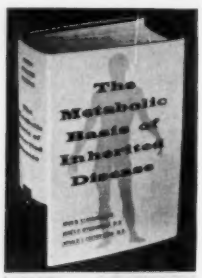

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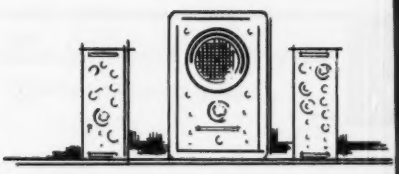

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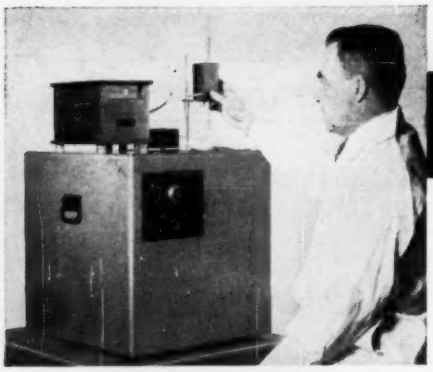

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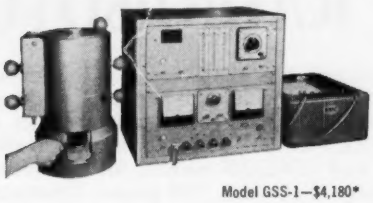

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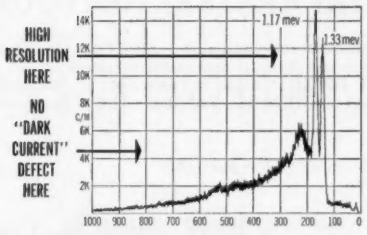

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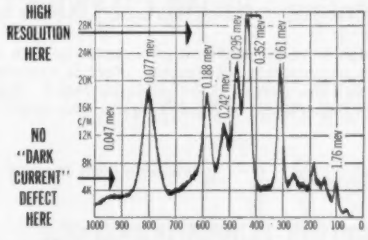

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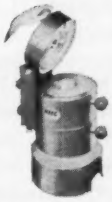

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As shown by animal studies, STAPHCILLIN is readily distributed in body tissues after intramuscular injection. Of the tissues studied, highest concentrations are reached in the kidney, liver, heart and lung in that order; the spleen and muscles show lower concentrations of the antibiotic. STAPHCILLIN diffuses into human pleural and prostatic fluids, but its diffusion into the spinal fluid has not yet been completely studied. However, one patient with meningitis showed a significant concentration in his spinal fluid while on STAPHCILLIN therapy.

Toxicity studies with STAPHCILLIN and penicillin G in animals show that they have approximately the same low order of toxicity.

Certain staphylococci can be made resistant to STAPHCILLIN in the laboratory, but this resistance is not related to their penicillinase production. During the clinical trials, no STAPHCILLIN-resistant strains of staphylococci were observed or developed; the possibility of the emergence of such strains in the clinical setting awaits further observation.

PRECAUTIONS

During the clinical trials, several mild skin reactions, e.g., itching, papular eruption and erythema were observed both during and after discontinuance of STAPHCILLIN therapy. Patients with histories of hay fever, asthma, urticaria and previous sensitivity to penicillin are more likely to react adversely to the penicillins. It is important that the possibility of penicillin anaphylaxis be kept in mind. Epinephrine and the usual adjuvants (antihistamines, corticosteroids) should be available for emergency treatment. Because of the resistance of STAPHCILLIN to destruction by penicillinase, parenteral *B. cereus* penicillinase may not be effective for the treatment of allergic reactions. Information with regard to cross-allergenicity between penicillin G, penicillin V, phenethicillin (Syncillin) and STAPHCILLIN is not available at present. If superinfection due to Gram-negative organisms or fungi occurs during STAPHCILLIN therapy, appropriate measures should be taken.

SUPPLY

List 79502 — 1.0 Gm. dry filled vial.

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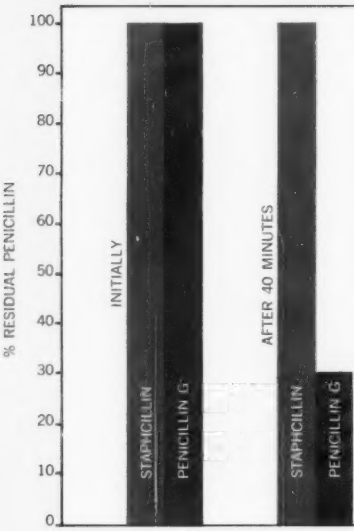

Specifically for

Stap

The failure of staphylococcal infe
the penicillin-destroying enzyme, p

Unlike other penicillins:

1 STAPHCILLIN is effective becau
ence of staphylococcal penicillinase

2 The clinical effectiveness of S
a wide variety of infections due to
and life-threatening.

Like other penicillins:

STAPHCILLIN has no significa
pain or irritation at the injection
penicillin G. *In occasional cases,*

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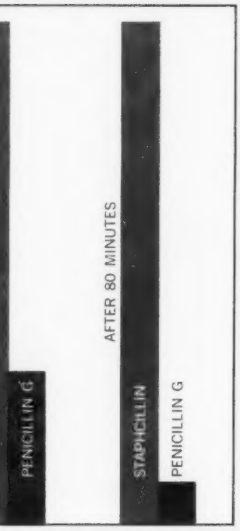

In the presence of staphylococcal penicillinase, STAPHCILLIN remained active and retained its antibacterial action. By contrast, penicillin G was rapidly destroyed in the same period of time.
(After Gourevitch et al., to be published)

or "resistant" staph...

phcillin™

sodium dimethoxyphenyl penicillin
FOR INJECTION

infections to respond to penicillin therapy is attributed to ne, penicillinase, produced by the invading staphylococcus.

ecause it retains its antibacterial activity despite the pres- inase.

of STAPHCILLIN has been confirmed by dramatic results in ue to "resistant" staphylococci, many of which were serious

ificant systemic toxicity. It is well tolerated locally, and tion site is comparable to that following the injection of *ses, typical penicillin reactions may be experienced.*

ON SERVICE – The attached Official Package Circular provides com- ns, dosage, and precautions for the use of STAPHCILLIN. If you desire clinical experiences with STAPHCILLIN, the Medical Department of ce. You may direct your inquiries via collect telephone call to New York, ical Department, Bristol Laboratories, 630 Fifth Ave., N. Y. 20, N. Y.

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UNIQUE SYNTHETIC "STAPH-CIDAL" PENICILLIN

OFFICIAL PACKAGE CIRCULAR
November, 1960

STAPHCILLIN™

(sodium dimethoxyphenyl penicillin)

For Injection

DESCRIPTION

STAPHCILLIN is a unique new synthetic parenteral penicillin produced by Bristol Laboratories for the specific treatment of staphylococcal infections due to resistant organisms. Its uniqueness resides in its property of resisting inactivation by staphylococcal penicillinase. It is active against strains of staphylococci which are resistant to other penicillins.

Each dry filled vial contains: 1 Gm. STAPHCILLIN (sodium dimethoxyphenyl penicillin), equivalent to 900 mg. dimethoxyphenyl penicillin activity.

INDICATIONS

STAPHCILLIN is recommended as specific therapy only in infections due to strains of staphylococci resistant to other penicillins, e.g.:

Skin and soft tissue infections: cellulitis, wound infections, carbuncles, pyoderma, furunculosis, lymphangitis and lymphadenitis.

Respiratory infections: staphylococcal lobar or bronchopneumonia, and lung abscesses combined with indicated surgical treatment.

Other infections: staphylococcal septicemia, bacteremia, acute or subacute endocarditis, acute osteomyelitis and enterocolitis.

Infections due to penicillin-sensitive staphylococci, streptococci, pneumococci and gonococci should be treated with Syncillin® or parenteral penicillin G rather than STAPHCILLIN. Treponemal infections should be treated with parenteral penicillin G.

DOSAGE AND ADMINISTRATION

STAPHCILLIN is well tolerated when given by deep intragluteal or intravenous injection.

As is the case with other antibiotics, the duration of therapy should be determined by the clinical and bacteriological response of the patient. Therapy should be continued for at least 48 hours after the patient has become afebrile, asymptomatic and cultures are negative. The usual duration has been 5-7 days.

Intramuscular route: The usual adult dose is 1 Gm. every 4 or 6 hours. Infants' and children's dosage is 25 mg. per Kg. (approximately 12 mg. per pound) every 6 hours.

Intravenous route: 1 Gm. every 6 hours using 50 ml. of sterile saline solution at the rate of 10 ml. per minute.

**Warning:* Solutions of STAPHCILLIN and kanamycin should not be mixed, as they rapidly inactivate each other. Data on the results of mixing STAPHCILLIN with other antibiotics are being accumulated.

DIRECTIONS FOR RECONSTITUTION

Add 1.5 ml. sterile distilled water or normal saline to a 1 Gm. vial and shake vigorously. Withdraw the clear, reconstituted solution (2.0 ml.) into a syringe and inject. The reconstituted solution contains 500 mg. of STAPHCILLIN per ml. Reconstituted solutions are stable for 24 hours under refrigeration.

For intravenous use, dilute the reconstituted dose in 50 ml. of sterile saline and inject at the rate of 10 ml. per minute.

*This statement supersedes that in the Official Package Circulars dated September and/or October, 1960.

(continued)

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ACUTE BRONCHITIS

SYNCILLIN
250 mg. t.i.d.

H.F. 45-year-old white female. First seen on Aug. 24, 1959 with acute bronchitis of 3 days' duration. Culture of the sputum revealed alpha hemolytic streptococci. A 250 mg. SYNCILLIN tablet was administered 3 times daily. Another sputum culture taken on Aug. 27 showed no growth. On Aug. 30, the patient appeared much improved and SYNCILLIN was discontinued.*
Recovery uneventful.

Illustrative case summary from the files of Bristol Laboratories' Medical Department

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Syncillin Tablets – 250 mg. (400,000 units)...Syncillin Tablets – 125 mg. (200,000 units)
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Syncillin Pediatric Drops – 1.5 Gm. bottles. Calibrated dropper delivers 125 mg. (200,000 units)

Streptococcal infections should be treated for at least 10 days to prevent the development of rheumatic fever and as prophylaxis against bacterial endocarditis in susceptible patients.

Complete information on indications, dosage and precautions is included in the circular accompanying each package.

BRISTOL LABORATORIES, Div. of Bristol-Myers Co., SYRACUSE, N.Y.

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1. Stephens, V. C., *et al.:* J. Am. Pharm. A. (Scient. Ed.), *48*:620, 1959.
2. Salitsky, S., *et al.:* Antibiotics Annual, p. 893, 1959-1960.
3. Reichelderfer, T. E., *et al.:* Antibiotics Annual, p. 899, 1959-1960.
4. Kuder, H. V.: Clin. Pharmacol. & Therap., in press.

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Lipid terminology should be specific—hypercholesterolemia, hypertriglyceridemia, etc. The nonspecific term "hyperlipemia" should be reserved for situations when all lipid moieties are elevated. The term "free fatty acids" is preferred to "unesterified fatty acids" or "nonesterified fatty acids."

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ABBREVIATIONS

absorbance	A
ampere	a
Ångstrom	Å
body surface area	BSA
boiling point	bp
centigrade	C
centimeter	cm
correlation coefficient	R
counts per minute	cpm
cubic centimeter	cc
curie	c
cycles per minute	c/min
cycles per second	c/sec
day	*sic*
Fahrenheit	F
free fatty acids (*not* UFA or NEFA)	FFA
gram	g
gram-centimeter	g-cm
gravity	G
half-time	$T_{\frac{1}{2}}$
hour	*sic*
inch	*sic*
inside diameter	ID
international units	IU
intramuscular	i.m.
intraperitoneal	i.p.
intravenous	i.v.
Kelvin	K
kilocycle	kc
kilogram	kg
kilogram-calorie	kg-cal
kilogram-meter	kg-m
kilometer	km
kilovolt	kv
kilowatt	kw
kilowatt hour	kw hr
lambda (λ)	μl
liter	L
melting point	mp
meter	m
microampere	μa
microcurie	μc
microequivalent	μEq
microgram	μg
microliter	μl
micromicron	μμ
micromolar	μM
micromole	μmole
micron	μ
microunit	μU
microvolt	μv
microosmole	μOsm
milliampere	ma
millicurie	mc
milliequivalent	mEq
milligram	mg
milligrams per cent	mg per 100 ml
milliliter	ml
millimeter	mm
millimicrogram	mμg
millimicromole	mμmole
millimicron	mμ
millimolar	mM
millimole	mmole
milliosmole	mOsm
millisecond	msec
milliunit	mU
millivolt	mv
minute	*sic*
molar	M
mole	*sic*
molecular weight	mol wt
month	*sic*
normal	N
not significant	NS
number	no.
optical density (see absorbance)	
outside diameter	OD
per	*sic*
per cent	*sic*
per os	p. o.
probability	p
regression coefficient	R
revolutions per minute	rpm
Roentgen	r
second	*sic*
sedimentation coefficient	s
sedimentation coefficient in water at 20°, extrapolated to zero concentration	$s^{\circ}_{20,w}$
standard deviation	SD
standard error	SE
subcutaneous	s.c.
Svedberg unit of sedimentation coefficient (10^{-13} sec)	S
"t" test	*t*
unit	U
versus	vs
volt	v
voltampere	va
volume (vol/vol)	vol
watt	w
weight	wt
year	*sic*
X-ray	*sic*

THE USE OF RADIOACTIVE CARBON DIOXIDE TO MEASURE REGIONAL BLOOD FLOW IN THE LUNGS OF PATIENTS WITH PULMONARY DISEASE

By J. B. WEST, C. T. DOLLERY and P. HUGH-JONES

(From the Department of Medicine, Postgraduate Medical School, Hammersmith Hospital, London, England)

(Submitted for publication August 10, 1960; accepted September 2, 1960)

The short-lived radioactive isotope, oxygen[15], can be used to measure the ventilation and blood flow in small regions of the lung without intubation of a patient (1, 2). The patient takes a single breath of air containing a minute amount of the active gas and breath-holds for 15 seconds, while scintillation counters aligned in front of and behind the chest detect the activity within the lung. The initial counting rate is determined by the ventilation of the lung core in the counting field. The fall in counting rate depends upon two factors: first, the transfer of the radioactive gas from alveolar gas to pulmonary capillary blood, and second, its removal from the counting field by the local blood flow.

When oxygen[15] is used as molecular oxygen, the initial transfer into the pulmonary capillary blood depends upon the local oxygen uptake (3). If the isotope is used to label carbon monoxide (4), its initial transfer is limited by the local diffusing properties of the lung. By converting the radioactive oxygen into carbon dioxide (5) which diffuses very readily, the transfer from alveolar gas to pulmonary capillary blood becomes exceedingly rapid. For all three gases, the fall in counting rate during the breath-holding period is influenced by the removal rate of blood from the counting field, but for carbon dioxide, this becomes the only significant factor. The present paper reports measurements made on 30 patients with various pulmonary diseases in whom regional blood flow was measured with radioactive carbon dioxide. The carbon dioxide measurements are also compared with the corresponding radioactive oxygen and carbon monoxide measurements in the same regions of the lung.

METHODS

Oxygen[15] was prepared in the Medical Research Council cyclotron in the grounds of Hammersmith Hospital by bombarding nitrogen with deuterons. The isotope has a half-life of only 2 minutes and it was therefore produced by a continuous flow process and pumped to a nearby clinical investigation room. Details of its preparation and purification have been published (1, 2). When the isotope was used as molecular oxygen, the gas was simply diluted about 20 times with air. To convert it into carbon monoxide, it was passed over heated carbon at 900° C, and any carbon dioxide formed was removed with soda lime. To prepare carbon dioxide, the gas was passed over heated carbon at 450° C and then over heated copper oxide at 800° C. The first process converted all the oxygen to a mixture of carbon dioxide and carbon monoxide, and the second removed the carbon monoxide. Periodical analyses for purity were made by using a mass spectrometer and an infrared analyzer.

The activity of the diluted gases was continuously monitored by a re-entrant ionization chamber and was approximately 5 mc per L. The maximal radiation dose to the blood or to any part of the body for 6 breaths of 1 L each is less than 400 mrads for radioactive oxygen and carbon monoxide, and less than 500 mrads for radioactive carbon dioxide. This dose is a little more than the weekly dose generally allowed for workers intermittently exposed to radiation but is small compared with the dose given by many special radiographic procedures such as screening, angiography and so forth.

The procedure was similar to that described previously (2). The patient was seated and took a rapid inspiration of 1 L of active gas from a plastic bag on the inspiratory side of a valve box with a low dead space. After 10 to 15 seconds of breath-holding he breathed room air, and the expired gas was piped from the room. Two pairs of counters were arranged, fore and aft, over each lung and the counting rates for the left and right pairs were displayed on two pen recorders. Figure 1 shows the tracings from a normal subject who inhaled a single breath of air containing radioactive carbon dioxide. The left-hand counters were at the level of the second rib, and those on the right were opposite the fifth rib anteriorly. The rapid initial deflection of the pens as the active gas entered the lung fields can be seen. The height of this initial deflection depends upon the length of the lung core in the counting field and on its ventilation. In this tracing, because the counters were at different levels where the lung cores had different length, the relative ventilations are not easily compared. In the measurements on the patients, both pairs of counters were placed

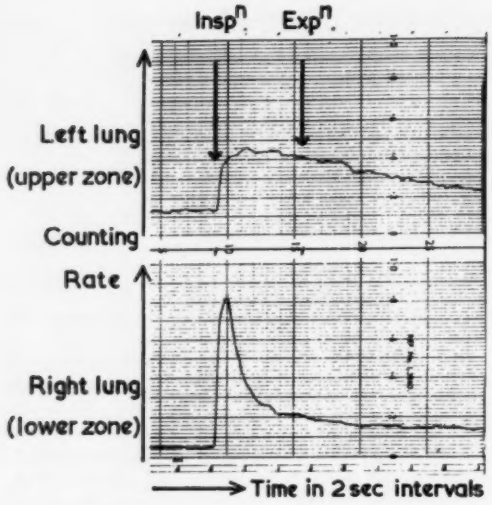

FIG. 1. TRACINGS FROM A NORMAL SUBJECT OBTAINED WITH RADIOACTIVE CARBON DIOXIDE. The left-hand counters were at the level of the second rib and those on the right were opposite the fifth rib anteriorly. The striking difference in blood flow between the upper and lower zones of the lung can be seen.

at the same vertical level and at the same distance from the sternum. When examining the upper lung zones, the counters were centered over the midclavicular line. For lower zone measurements, the counter centers were separated by about 18 cm in order to exclude the heart from the counting field as far as possible. When the counters were symmetrically placed in this way, the height of the initial deflection was taken to be proportional to the ventilation of the lung core examined.

During the breath-holding period, the counting rate fell on both sides but the very slow fall at the left upper zone contrasts with the rapid fall at the right lower zone. The rate of fall is taken to be proportional to the blood flow of the lung core examined, and it can be shown that this steadily increases as the counters are moved down the normal chest (5). The fall in counting rate of carbon dioxide and carbon monoxide is exponential so that the slopes were replotted on semilogarithmic paper and the clearance rates expressed in per cent per second of the instantaneous activity. Appropriate corrections were made for the background and the natural decay of the isotope. The oxygen tracings have an approximately linear slope during the breath-holding and the clearance rate was expressed in per cent per second of the initial counting rate.

The time constant of a pair of counters with its amplifiers and pen recorder was 0.5 second and the ratemeter integrating time was 0.2 second. All the measurements were made with the counters connected in parallel, when the anteroposterior cylindrical core of lung from which 95 per cent of the counts was accepted was 9 cm in diameter (Figures 3–7). A smaller counting field can be effected by connecting the counters in coincidence, but then the lower counting rates result in poorer repeatability of the measurements.

In order to define normal values for all 3 gases on the same group of subjects measurements were made on 10 healthy volunteers who were members of the hospital staff. Six breaths on each subject were given, and in all, 4 vertical counter positions were used. These were at the level of the second rib anteriorly and 2, 4 and 5 inches below this. The first breath on one normal subject was excluded because the radioactive carbon dioxide clearance rates were considerably higher than in the second breath when the subject's pulse rate had slowed (see Discussion). The results are summarized in Table I.

Thirty patients with various pulmonary diseases were studied. In 4 patients the pulmonary vessels were primarily affected by emboli, thrombosis or unknown causes. Ten patients had localized cysts, bullae or areas of transradiancy, and there were 2 cases of unilateral transradiancy as described by Macleod (6). A further 10 patients had localized fibrosis caused by old infection, radiation or pneumoconiosis. Cases of bronchial carcinoma, pleural effusion and bronchiectasis also were studied.

RESULTS

Figure 2 shows a tracing obtained from a 12 year old boy who had clinical and angiographic evidence of an absent left pulmonary artery. In addition he had a ventricular septal defect. The counters were placed at the level of the second intercostal space and each pair was 7 cm from the mid-line. The rapid initial deflections of the pens as the patient breathed-in the radioactive carbon dioxide can be seen. Since the cores of the lung tissue in the counting fields were of the same length, the initial counting rates were proportional

TABLE I

Clearance rates of radioactive carbon dioxide, oxygen, and carbon monoxide in 10 healthy volunteers measured in the sitting position

	Carbon dioxide	Carbon monoxide	Oxygen
	%/sec	%/sec	%/sec
Second rib anteriorly	4.8 (2.1)	2.9 (0.8)	0.5 (0.1)
Below second rib, 2 in.	12.0 (1.6)	6.0 (0.6)	1.2 (0.3)
Below second rib, 4 in.	23.5 (1.7)	8.9 (0.8)	2.2 (0.2)
Below second rib, 5 in.	24.8 (1.5)	10.2 (0.5)	2.6 (0.2)

* Means and standard error of mean (in parentheses) are given.

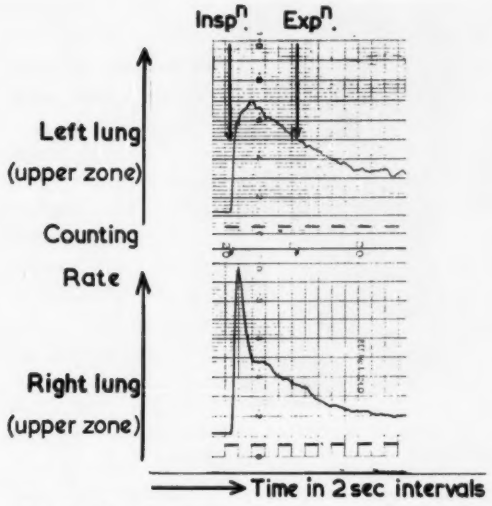

FIG. 2. TRACINGS FROM A PATIENT WITH ANGIOGRAPHIC EVIDENCE OF AN ABSENT PULMONARY ARTERY ON THE LEFT, AND A VENTRICULAR SEPTAL DEFECT. The counters were at the level of the second intercostal space anteriorly. The slow clearance on the left is contrasted with the rapid clearance on the right, where recirculation of labeled blood interrupted the curve 3 seconds after the beginning of inspiration.

to the ventilation in each lung core. In this case, the relative ventilations, left to right, were 0.6 to 1. (In practice, radioactive oxygen rather than carbon dioxide is used for the ventilation measurements because it is removed much more slowly, and any error caused by slow inspiration is thus minimized.)

During the breath-holding period, the radioactive carbon dioxide was removed very rapidly on the right side, and the calculated clearance rate was 38 per cent per second, compared with the normal (adult) value of about 7 per cent per second at this level. This high value is explained by the passage of the whole cardiac output through the right lung augmented by the left to right shunt. Some 3 seconds after the beginning of inspiration, the rapid fall in counting rate on the right was interrupted by a transient rise in activity. This was caused by the recirculation of labeled blood through the lung and its early appearance is explained by the intracardiac shunt.

On the left the counting rate fell slowly. Close inspection shows that this occurred only after recirculation had begun and that during the first 3 seconds of breath-holding, the counting rate actually rose slightly, probably because of increasing radioactivity in the chest wall and bronchial circulations. The tracing suggests that the pulmonary blood flow was absent or very small.

At the end of the breath-holding period, any radioactivity remaining in the alveolar gas is washed out as the patient breathes room air. The counting rate which remains is due to radioactive blood and tissue.

Figure 3 shows the angiocardiogram of a 29

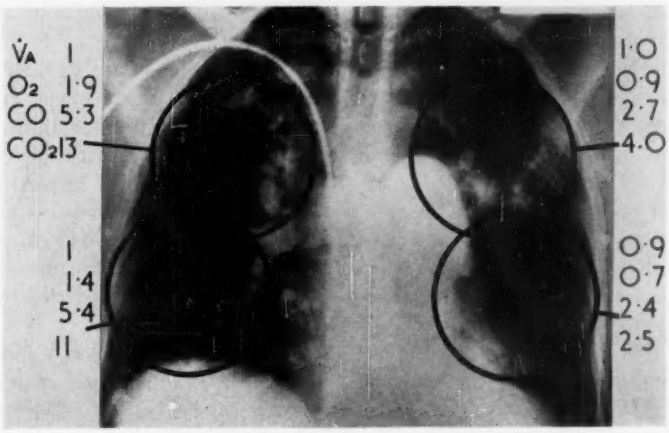

FIG. 3. ANGIOCARDIOGRAM OF A PATIENT WITH MULTIPLE PULMONARY EMBOLI. Circles show the lung cores from which counts were accepted. The figures are the radioactive carbon dioxide, carbon monoxide and oxygen clearance rates, and the relative ventilation (left to right) in each zone.

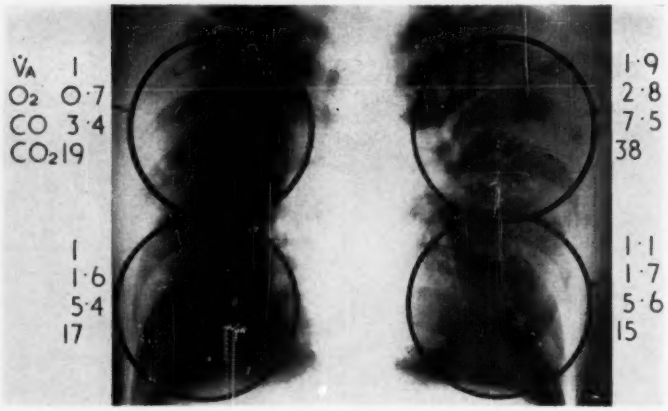

FIG. 4. ANGIOGRAM OF PATIENT WHO WAS THOUGHT TO HAVE A REDUCED BLOOD FLOW IN THE RIGHT LUNG. The radioactive measurements suggested, however, that the left lower lobe was also diseased, and review of the angiogram confirmed this.

year old engineer who had developed thrombophlebitis of the left calf 6 months previously, and had complained of a second episode in the left thigh 3 months before. He was admitted to hospital because of progressive shortness of breath on exertion. On several occasions he had coughed up clots of blood in the morning. The angiocardiogram was reported by Dr. R. Steiner as showing "gross enlargement of the main pulmonary artery and of the right and left branches. A few relatively normal caliber vessels were seen going into the right lower lobe, but elsewhere in the right lung, the peripheral vessels were of very narrow caliber and irregular in their course. There was virtually no arterial filling at the apex of the right lung. The left lung showed no filling whatsoever in the left lower zone, and sparse filling in the left upper zone. The appearances are consistent with multiple arterial thromboses and complete thrombosis of the main left lower lobe."

Measurements using radioactive carbon dioxide were made 6 days after the angiocardiogram when the clinical condition was unchanged. The counters were centered over the second rib anteriorly and 3 inches below this on both sides of the chest, and circles have been drawn on the radiograph to show the cylindrical cores of lung from which counts were accepted. The clearance rates of radioactive carbon dioxide, carbon monoxide and oxygen and the relative ventilations (right side taken as unity) are also shown on Figure 3. It can be seen that the clearance rates of all three gases were greatly reduced in the left lung compared with the right, although the ventilations on the two sides were comparable. The reduction in blood flow was apparently less marked in the left upper zone (carbon dioxide clearance rate 4.0 per cent per second) than in the left lower zone (2.5 per cent per second). Of the four zones examined, the right upper had the best function.

In general, the radioactive carbon dioxide clearance rates and the angiocardiogram indicated similar blood flow distributions. Both showed a very low blood flow at the left base and impaired perfusion of the left upper zone. The only discrepancy was the high clearance rate in the right upper zone where the angiocardiogram was reported as showing no filling at the right apex. This apparent disagreement was resolved when the films were examined because, whereas the extreme apex of the right lung received no dye, the upper zone in the region of the second rib was well perfused (Figure 3). The carbon monoxide and oxygen clearance rates correlated closely with the carbon dioxide clearance rates.

Good agreement between the radioactive carbon dioxide clearance rates and the angiogram was also seen in the case of a 29 year old nurse who had complained of increasing breathlessness on exertion, cough, and purulent sputum for many years. A diagnosis of unilateral transradiancy (6) on the right side had been made at another

hospital because an angiogram showed reduced vascularity on that side. At thoracotomy, the surgeon reported some collapse of the main and lobar bronchi on the right and these were stiffened with wire. Some improvement apparently followed this, although the patient's mental state made objective assessment difficult. Her breathlessness subsequently increased and the possible benefit from right pneumonectomy was being considered when she was referred for assessment.

Measurements were made with the counters at the level of the second intercostal space and fourth rib anteriorly, and the results are summarized in Figure 4. They show that the clearance rates of radioactive carbon dioxide, carbon monoxide and oxygen, and the ventilation, were all greatly reduced in the right upper zone compared with the left. The function of both lower zones was very similar. The higher clearance rates in the left upper zone compared with both lower zones suggested that the latter also were diseased. When the angiogram films were re-examined, it became clear that the filling of the left lower lobe, as well as that of the right lung, was defective and that the apparently normal pattern at the left base had been caused by vessels in the lingula. In fact, the only well perfused part of the lung was the left upper lobe, and the correlation with the radioactive measurements was agreed to be excellent. Right pneumonectomy was not advised.

Figure 5 shows the radiograph of a 46 year old deputy foreman who complained of cough and progressive shortness of breath. Measurements were made with the counters at the level of the second and fifth ribs anteriorly and show that no gas entered the large cyst in the right lower zone. The clearance rates in the upper zones were of interest in that they were much increased (for example, the radioactive carbon dioxide clearance rates were both 18 per cent per second compared with the normal value of 4.8 per cent per second at this level). This is the usual finding in the presence of a large lesion elsewhere in the lung and presumably indicated a "compensatory" increase in local blood flow, when blood which had been diverted from the diseased area had opened up more capillaries.

This increased function in an apparently normal area in the presence of disease elsewhere in the lung makes the application of the normal data of Table I difficult in some instances. An example was a 40 year old slater and tiler who complained of dull pain in the left chest but no shortness of breath. He had a large cyst in the left upper zone occupying almost half of the left hemithorax, and the results of the measurements are summarized (Patient 1) in Table II. It can be seen that the clearance rates of radioactive carbon dioxide, carbon monoxide, and oxygen (3.6, 1.9, and 0.4 per cent per second, respectively) in the

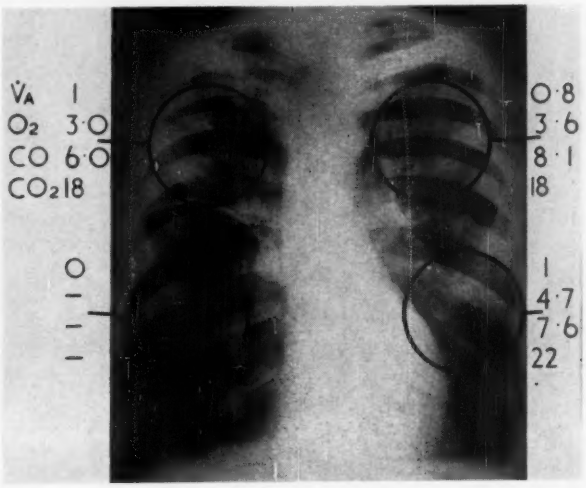

FIG. 5. RESULTS FROM A PATIENT WITH LARGE CYST IN THE RIGHT LOWER ZONE.

TABLE II

*Summary of the radioactive measurements in 10 patients**

Patient	Age	Sex	Diagnosis	Radiological change	Counter positions	Radioactive measurements								Comments
						CO_2		CO		O_2		$\dot{V}_A$		
						R	L	R	L	R	L	R	L	
1	40	M	Solitary lung cyst	Large cyst left upper zone	Second rib	3.6	0	1.9	0	0.4	0	1	0	No gas entered left upper zone
					4 in. below	27	24	6.0	4.3	3.1	2.8	1	1.2	
2	31	F	Tuberculoma?	Opacity at right hilium; reduced vascular markings in surrounding lung	Second rib	3.1	6.1	0.5	2.9	0.8	1.0	1	1.8	Reduced gas exchange in right lung
					4 in. below	16	24	4.2	7.3	1.9	2.6	1	0.7	
3	40	M	Pulmonary fibrosis	Mottled lower zones; "emphysematous" upper zones	Second rib	3.3	2.2	0	0	0	0	1	0.7	Reduction in all areas
					fifth space	10	5.7	1.8	0.8	1.0	1.2	1	0.8	
4	35	F	Lymphangitis carcinomatosa	Fine fibrosis in right lung	Second rib	7.0	19	4.6	6.4	1.7	3.0	1	1.5	Reduced function on right
					3.5 in. below	10	21	3.8	8.1	1.5	3.5	1	1.7	
5	52	M	Lung abscess?	Lung opacity in right	Second rib	24	33	5.9	6.8	1.2	2.7	1	1.5	No gas exchange in right lower zone
					3 in. below	0	34	0	6.4	0	3.7	1	7	
6	55	M	Breathlessness and cyanosis; cause?	Mottling in all areas	Second rib	18	9.3	0	0.3	1.0	0.6	1	1.6	CO_2 clearance reduced in lower zones
					4 in. below	6.9	5.9	0.8	0.1	1.3	0.9	1	1.0	
7	39	M	Unilateral transradiancy	Transradiant left lung	Second rib	23	0	44	0	1.4	0	1	0.3	Reduced function on left
					4 in. down	46		9.1	0	5.2	0	1	0.3	
8	64	M	Unilateral transradiancy; emphysema?	Transradiant left lung	Second rib	13	0	1.9	0	0.6	0	1	0.4	Reduced function on left
					fourth space	19	0	3.4	0	3.3	0	1	0.4	
9	36	M	Localized transradiancy	Transradiant area right lower zone	Second rib	2.4	3.4		3.0	0.4	0	1	1.1	Reduced function right lower zone
					fourth space	11.5	14.8	8.2	10	1.3	1.7	1	1.6	
10	20	M	Unilateral transradiancy	Transradiant left lung	Second rib	18	28	5.8	6.3	1.7	1.4	1	1.8	Reduced function left lower zone
					fourth space	33	20	9.9	7.1	3.7	2.4	1	0.5	

* The clearance rates are in per cent per second, and relative ventilation is left to right with the latter scaled to unity.

right upper zone were relatively normal according to Table I. However, because of the lack of "compensatory" increase in the right upper zone in the presence of such a large lesion on the left, tomographs of the right upper lobe were ordered. These showed further small cysts and the failure of the clearance rates to increase in this area was then explained. Thus the finding of "normal" clearance rates (on the criteria of Table I) was here evidence for local disease.

A different functional pattern in the presence of cysts is illustrated by Figure 6 which shows the radiograph of a 19 year old teleprinter operator. She had no respiratory symptoms but was referred following a mass miniature radiograph which showed large air-containing cysts in the right mid and lower zones of the chest. The results indicate that the ventilation in the right lower zone was greatly reduced compared with that in the left lower zone (ratio, 1 to 2.5). However, the clearance rates of carbon dioxide, carbon monoxide and oxygen were only slightly less on the right than the left. This disparity between the ventilation and clearance rates is compatible with a large nonventilating lesion at the right base surrounded by a thin shell of lung which had almost normal function. In these circumstances the initial counting rate would be grossly reduced, but the clearance rates which depend upon the blood flow and gas exchange per unit volume of ventilating lung would be normal. In the upper zones there were "compensatory" increases in clearance rates, less marked on the right where the ventilation was reduced, presumably because of interference by the cysts.

Figure 7 shows the bronchogram of a 39 year old housewife who had severe saccular bronchiectasis of the right lung with possibly some disease on the left. She had complained of much sputum and shortness of breath for many years and had suffered from recurrent respiratory infections. Pneumonectomy was contemplated and she was referred to establish the respiratory value of the right lung.

Measurements were made with the counters at the level of the second intercostal space and fifth rib anteriorly. The results show that the function of the right lower zone was grossly impaired. The clearance rates of radioactive carbon dioxide, carbon monoxide and oxygen were all exceedingly low, and the ventliation was about one-half that of the left lower zone. The function in the right upper zone was considerably less than that in the left upper zone, although the figures were not low by comparison with Table I. There is no doubt that this part of the lung was diseased as evi-

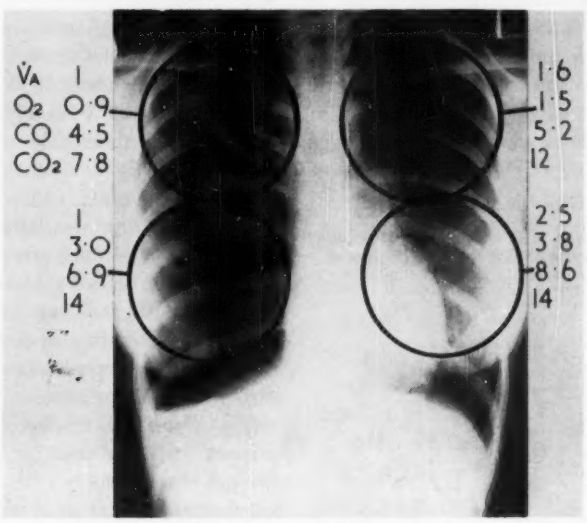

FIG. 6. RESULTS FROM A PATIENT WITH LARGE CYSTIC AREA IN THE RIGHT MID AND LOWER ZONES.

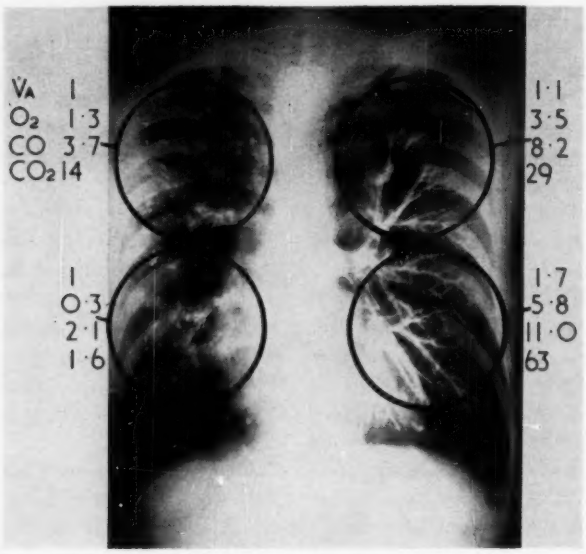

FIG. 7. BRONCHOGRAM OF PATIENT WITH SEVERE BRONCHIECTASIS IN THE RIGHT LUNG.

denced by the bronchial dilatation shown on the bronchogram, but apparently sufficient lung reserve had opened up to give normal clearance rates. The clearance rates on the left side were all greatly increased.

Table II summarizes the findings on nine other patients on whom measurements were made in four zones of the lung using all three gases.

In order to determine the repeatability of the clearance rate measurements, 15 pairs of duplicate breaths for each of the three gases were analyzed in 12 consecutive patients who had been referred for examination, and the results are shown in Table III. In the normal use of oxygen[15] some of the curves obtained are unsatisfactory, perhaps because the patient failed to empty the bag completely, or because he moved during the breath-holding period, or for some other technical reason. Any such cases which would normally have been repeated (a very simple process for this test) were excluded from the repeatability series, the aim of which was to determine the reliability of measurements routinely accepted. Duplicate breaths for all three gases on the ten normal volunteers were also analyzed and the results are also shown in Table III. One breath was excluded, this being the first breath in a subject who showed initially high clearance rates for radioactive carbon dioxide which settled to normal values after a few minutes' rest in the chair (see Discussion).

TABLE III

Repeatability of clearance rates in 12 consecutive patients and 10 normal volunteers expressed as the average deviation of duplicates from their means (as a percentage)

	Radio-active carbon dioxide	Radio-active carbon monoxide	Radio-active oxygen
Normal subjects (all counting positions except second rib level)	7.2	7.1	12.6
Normal subjects (second rib level)	35.8	14.7	36.0
Patients (all counting positions)	6.7	10.7	11.2

It can be seen that in both patients and normal subjects, the repeatability of the radioactive carbon dioxide clearance rates was best, followed by the carbon monoxide and oxygen measurements. The reason for the larger variation in the oxygen clearance rates is that the slopes are small, and the statistical fluctuations in counting rate therefore cause more uncertainty when the line of visual best fit is drawn. Such counting

rate fluctuations become increasingly less important as the clearance rate increases. Dr. C. Matthews has calculated by an approximate method that for a counting rate of 2,000 counts per second, a ratemeter integrating time of 0.2 second and a breath-holding period of 10 seconds, the standard deviation of the slope of a line of best fit about a true slope of 2 per cent per second would be 0.2 per cent per second. This calculation does not include the effect of the time constant of the recording pen.

DISCUSSION

As a method for measuring regional blood flow in the lung, the use of radioactive carbon dioxide compares favorably with angiography or bronchospirometry. From the patient's point of view the test is simple and quick, and there is no associated discomfort. There are none of the obvious disadvantages of catheterization or intubation, and the only unphysiological feature is 10 seconds of breath-holding. As yet no patient has been so breathless that he could not cooperate satisfactorily. The measurements can easily be repeated at intervals, and the radiation hazard is small by the standards of diagnostic radiology.

An important practical limitation is that the oxygen[15] must be prepared in the vicinity because it is so short-lived. While a cyclotron is not essential for this, a powerful particle accelerator or source of X-rays is necessary, and such expensive equipment is only likely to be available in the larger centers.

It is true that in many instances, a plain chest radiograph will give valuable information about regional blood flow. There may be an obvious area of fibrosis, for example, or vascular markings may be reduced. However, such appearances can be very misleading. In Patient 4 of Table II, the radiograph showed a very fine fibrosis on the right which was not at all obvious. This was thought at the time to be an early postradiation fibrosis, although the subsequent course proved it to be lymphangitis carcinomatosa following carcinoma of the breast. The radioactive carbon dioxide measurements showed that the blood flow had in fact been reduced to less than one-half, compared with the normal side.

Another example of poor correlation between radiographic appearances and the clearance rates was Patient 10 of Table II. This 20 year old National Serviceman had no symptoms, but his chest radiograph showed obvious transradiancy of the whole of the left lung with diminished vascular markings in both upper and lower zones. As expected, the clearance rate of radioactive carbon dioxide was reduced in the left lower zone compared with the right, but surprisingly, the reverse was the case for the upper zones. The carbon monoxide clearance rate and ventilation were also higher in the left upper zone than in the right. It is difficult to explain these findings, which were confirmed on repeated observations, but it is clear that an area of the lung which appeared abnormal on the radiograph had, in fact, excellent function.

In some instances, the radiograph may show disease in several parts of the lung, and to assess the relative function on appearances alone may be impossible. Patient 3 was a 40 year old railwayman who had considerable disability, and whose radiograph showed a striking mottling of both lower zones and "emphysematous" upper zones. He was referred so that the relative contribution of the upper and lower zones (all of which had obvious disease) could be determined. The measurements showed that the pulmonary blood flow was reduced in all areas but that the right lower zone was the best of the four.

On theoretical grounds, the clearance rates of radioactive carbon dioxide will be affected by variations in cardiac output, and there is good evidence that excitement or apprehension causes high values. Exercise in normal subjects raises the clearance rates in the upper zones faster than in the lower zones (5), and the commonest artifact caused by nervous tachycardia is therefore unduly high clearance rates in the upper zones. As an example, the radioactive carbon dioxide clearance rate for the first breath of one of the normal subjects was 18 per cent per second at the level of the second rib, and 31 per cent per second 4 inches lower down. At this time his pulse rate was 106 per minute. The corresponding clearance rates after a few minutes' rest in the chair were 4.2 and 13.6 when his pulse rate had fallen to 84. Such large variations are unusual but may cause considerable confusion unless care is taken to ensure a relatively basal state. It is possible that the

high clearance rates seen in some cases (for example Figures 4 and 7, and Patients 5 and 10 in Table II) may reflect high cardiac output resulting from excitement, or result from insufficient time allowed for the patient to recover from walking to the clinical investigation room. In order to assess regional lung function in these instances, less reliance must be placed upon comparisons with the normal data of Table I than on relative clearance rates in different zones.

In some patients on whom measurements have been made, the clearance rates of radioactive carbon dioxide tended to be low in all areas of the lung. While this has been particularly common in patients with emphysema (which will be described elsewhere), Patient 3 in Table II and the patient whose angiocardiogram is shown in Figure 3 are examples. Inhaled radioactive carbon dioxide can be cleared only by pulmonary blood which is perfusing ventilated alveoli, so that appreciable shunting of blood through nonventilated lung may result in low carbon dioxide clearance rates. However, a more probable explanation in these patients is that there is so much blood flow inequality within the counting field that the shallow slopes of the poorly perfused areas predominate in the over-all clearance rate, although it can be shown that considerable inequality is necessary before this effect is appreciable. A general reduction in the clearance rates of radioactive carbon monoxide is associated, as would be expected, with a low over-all carbon monoxide diffusing capacity as measured by the single-breath test (7). For example, in Patient 6 of Table II, the carbon monoxide clearance rates were reduced in all areas and the over-all diffusing capacity was down to one-quarter of the expected value. A high correlation between regional and over-all carbon monoxide uptakes has also been seen in other patients with diffusing defects of the lung.

A feature of the results is the correlation in each subject between the clearance rates of the three gases in the four lung zones examined. The results set out in Figure 3 are a good example of this. The carbon dioxide clearance rate is highest in the right upper zone and then follows the right lower zone, left upper zone, and left lower zone in that order. Precisely the same order applies to the carbon monoxide and oxygen clearance rates. Other good examples can be found in Table II.

This correlation is not surprising when it is appreciated that the loss of the inhaled isotope from the counting field occurs in two stages. First, the tracer must be transferred from the alveolar gas to the pulmonary capillary blood, and second, the isotope must be removed by the local blood flow. In earlier work with oxygen[15], it was thought that the fall in counting rate during breath-holding chiefly gave information about the initial transfer, so that the oxygen clearance rate was taken as a measure of the local oxygen uptake (1) and the carbon monoxide clearance rate as a measure of the local diffusing properties of the lung (4). However, recent work has shown that the second stage—that is, the rate of removal of the tracer from the counting field by the local blood flow—is an important factor for all three gases so that all of the clearance rates are weighted by blood flow (8).

Oxygen[15]-labeled carbon dioxide is a particularly valuable gas, because its transfer from alveolar gas to capillary blood is exceedingly rapid. For this reason its clearance rate is, in practice, solely determined by the rate of removal of the blood from the counting field. The carbon dioxide clearance rate can therefore be used as a measure of the second stage for both oxygen and carbon monoxide, and this enables the initial stage—that is, the rate of transfer from alveolar gas to capillary blood—to be calculated (8).

When the calculations are made for each lung zone, using the data in Table II, the variations in radioactive carbon monoxide clearance rate can be shown, in many cases, to be caused not by differences in the initial transfer from alveolar gas, but by differences in the rate of removal of the blood from the counting field. In other words, the clearance rate variations are not reflecting inequality of local diffusing properties so much as differences in local blood flow. For example, in Figure 3, the carbon monoxide clearance rates on the right were 5.3 and 5.4 per cent per second compared with 2.7 and 2.4 per cent per second on the left. By using the appropriate carbon dioxide clearance rates to extract the blood flow components, the alveolar disappearance rates on the right become equal to about 10 per cent per second,

while on the left, the rates are even higher. Thus, the measured clearance rates chiefly reflect blood flow differences. Another example is Patient 1 in Table II in whom the carbon monoxide clearance rates in the right upper and lower zones were 1.9 and 6.0 per cent per second, respectively. After allowing for the large difference in blood flow in the two areas (carbon dioxide clearance rates of 3.6 and 27.6 per cent per second), the rates of transfer of carbon monoxide from the alveolar gas in the two zones become approximately equal (10 and 7 per cent per second).

The calculations for radioactive oxygen are less reliable, but the analysis using carbon dioxide clearance rates suggests that, in many instances, inequality of oxygen transfer from alveolar gas does remain. Indeed, this would be expected, since oxygen uptake is generally flow-limited.

The conclusions about the true inequality of carbon monoxide and oxygen transfer from alveolar gas must be regarded as tentative, because the calculations use two poorly repeatable measurements. However, the general correlation of the clearance rates of carbon dioxide, carbon monoxide and oxygen is easily appreciated in that all three are partly measuring blood flow.

Although in general the clearance rate of all three gases is reduced in an area of local disease, there are instances in which, for example, the radioactive carbon monoxide clearance rate is reduced out of proportion to the carbon dioxide clearance rate. Patient 6 in Table II was a 55 year old electrician who was referred because of breathlessness and cyanosis on exercise. The radiograph showed diffuse mottling in all areas, with little evidence of localized disease. The radioactive measurements indicated reduced ventilation in the right upper zone where there was no measurable carbon monoxide clearance although the carbon dioxide clearance rate was high. These findings were confirmed on repeated measurements and suggest a diffusing defect in a well perfused area caused, perhaps, by a thickened alveolar membrane. In addition there was a generalized reduction in radioactive carbon monoxide clearance rates and a low diffusing capacity measured by the single-breath test (7 ml per minute per mm Hg; normal range 21 to 36), all pointing to impaired diffusion.

SUMMARY

Carbon dioxide labeled with oxygen[15] has been used to measure regional pulmonary blood flow in a series of 30 patients with a variety of lung diseases. The isotope has a half-life of only 2 minutes and is prepared in a cyclotron in the hospital grounds. For the patient, the measurement is very simple and the radiation dose is small. He takes a single breath of air containing a small quantity of active gas and breath-holds for 15 seconds. Scintillation counters placed in front of and behind the chest detect the activity of gas within the lung, and the clearance rate during breath-holding is a measure of the pulmonary blood flow in the lung core examined.

Good correlation was found between the radioactive measurements and angiograms in four patients, but the plain radiographs showed some interesting discrepancies in a further group. In most cases the radioactive carbon dioxide clearance rates were found to be closely related to radioactive oxygen and carbon monoxide clearance rates. Striking increases in local blood flow were found in apparently normal areas in the presence of disease elsewhere in the lung, and this complicates a comparison with clearance rates determined from normal subjects. The use of radioactive carbon dioxide for measuring regional blood flow compares favorably with the traditional techniques of angiography and bronchospirometry.

ACKNOWLEDGMENTS

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A LABILE SERUM FACTOR CLOTTING DEFECT: ITS DEMONSTRATION BY THE THROMBOPLASTIN GENERATION TEST AND ITS CLINICAL SIGNIFICANCE *

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(Submitted for publication August 3, 1960; accepted August 19, 1960)

The thromboplastin generation test, originally described by Biggs and Douglas (2), has proved to be a valuable tool in the study and diagnosis of hemorrhagic disease. Several problems have arisen from its use, however. These have centered about the preparation of the reagents for the test and the implications of an abnormal test result. This test, which measures the rate of formation and the amount of plasma thromboplastin, may be performed with platelet factor 3 supplied by either a platelet suspension or an emulsion of phosphatides from soybeans or brain. In the routine performance of the test we have used a soybean phosphatide.

Early in the course of our studies with the thromboplastin generation test, we occasionally found a serum factor deficiency different from deficiencies of PTC (factor IX) or Stuart factor (factor X). This labile serum factor defect was not evident in fresh serum but developed as the serum was aged from 3 to 24 hours. The abnormality was noted in some patients with undiagnosed bleeding whose other coagulation tests were normal. Patients with liver disease, patients with polycythemia vera, and newborn infants usually had this defect. It was sometimes found, combined with other defects, in patients receiving coumarin drugs. It occured in a few "normal" control subjects. Such defective thromboplastin generation could be corrected readily by the use of any one of the following in the test system: serum aged 3 to 24 hours from most normal human subjects; serum aged for only 1 or 2 hours from the patient under study; aged sera from patients with PTC (factor IX) deficiency; sera deficient in Stuart factor (factor X); and by the use of platelets instead of soybean phosphatide as the source of platelet factor 3. Some characteristics of this labile serum factor suggest a similarity to the factor in serum described by Duckert, Flückiger, Matter and Koller (3) and Spaet and Kropatkin (4).

The studies to be reported will illustrate and define this labile serum factor and will point out its possible significance in patients.

METHODS

All blood was drawn by the two-syringe technic. Normal oxalated plasma was obtained by adding 7 ml of whole blood to 1 ml of 1.85 per cent potassium oxalate solution and centrifuging the mixture 10 minutes at 4,000 rpm and 4° C. Adsorbed plasma was provided by mixing 1 ml of oxalated plasma with 100 mg barium sulfate for 30 minutes with a magnetic stirrer and then centrifuging. Such plasma did not clot after the addition of brain thromboplastin and calcium chloride, and its prothrombin content by the two-stage method was always less than 5 per cent and usually unmeasurable. Barium sulfate-adsorbed serum was prepared similarly. For the "aged serum" reagent, blood was allowed to clot and incubated for 1 hour at 37° C. The serum was then removed and aged additionally at 37° C for variable periods of time. Most studies were performed with reagents which had not been frozen previously. We did demonstrate, however, that sera which in the fresh state had the labile serum factor defect continued to manifest the same defect when stored at −30° C for as long as 2 weeks before being thawed and tested. The age of the serum was designated as the number of hours of incubation after venipuncture. The soybean phosphatide [1] concentration was 0.1 per cent in saline. A platelet suspension was prepared from platelet-rich plasma obtained from blood centrifuged at 700 rpm in an angle centrifuge at 4° C. Platelets were then separated by centrifugation of the platelet-rich plasma at 4,000 rpm. The resulting

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[1] Gliddex "O" (Glidden Co.).

"platelet" clump was broken up and washed 3 times with saline. After the final resuspension in a volume of saline equal to the volume of the plasma from which they were derived, the platelets were frozen and thawed 3 times prior to use to insure adequate disruption. Thrombin at a strength of 100 U per ml was prepared from bovine thrombin.[2] It was diluted with normal saline to give various concentrations. This thrombin-saline solution was employed as the diluent for the serum or barium sulfate-adsorbed plasma whenever thrombin was to be added to the thromboplastin generation test.

The thromboplastin generation test (TGT) (5) was performed by incubating 0.3 ml $BaSO_4$-adsorbed plasma (diluted 1/5 with saline), 0.3 ml aged serum (diluted 1/5 with saline), 0.3 ml of 0.1 per cent soybean phosphatide, and 0.1 ml of 0.015 M $CaCl_2$. The adsorbed plasma and the aged serum were used immediately after dilution. Plasma having a normal concentration of prothrombin and fibrinogen was used for the substrate or indicator plasma. When thromboplastin generation was normal, a fibrin clot always formed in the incubation tube at about the time maximal thromboplastin developed. No clot formed in the incubation tube when thromboplastin generation did not occur.

Corrections of abnormal test results were made as follows. The serum, which was to be tested for corrective potential, was supplied to the system in place of 0.15 ml of the patient's diluted serum, so that the total serum reagent volume was maintained at the usual 0.3 ml and consisted of 0.15 ml of the patient's diluted serum and 0.15 ml of the diluted "corrective" serum. Corrective sera included normal serum, the patient's serum aged for shorter periods of time, PTC (factor IX) deficient serum, and Stuart factor (factor X) deficient serum.[3] In some tests the platelet suspension was used to replace the soybean phosphatide as the source of platelet factor 3. $BaSO_4$-adsorbed plasma from normal subjects was employed in place of the patient's adsorbed plasma. The corrective effect of thrombin was tested by supplying it to the thromboplastin generation test as the thrombin-saline diluent for the serum or $BaSO_4$-adsorbed plasma. More highly purified bovine thrombin and prothrombin[4] were added in the same way for use in TGT correction.

To express thromboplastin generation capacity in a numerical value related to percentage of normal, the area above the thromboplastin generation curve was compared with that obtained with the blood of normal controls. The clotting times of the substrate plasma were plotted against the incubation time of the thromboplastin generation mixture. The area encompassed by the curve with the limits of a 60 second clotting time and a 2 to 12 minute incubation period was then measured with a planimeter. This area is dependent upon both the amount of thromboplastin generated and the rate of its formation. A test result below 75 per cent was considered definitely abnormal. Most of the patients with the labile serum factor defect had values from 0 to 20 per cent.

Thrombin and prothrombin were measured by two-stage methods (6, 7) and antithrombin by the method of Seegers, Miller, Andrews and Murphy (8).

Patients described in this study included several groups: those with unexplained clinical bleeding referred for diagnostic study; patients with liver disease; patients with polycythemia vera; newborn infants; and patients receiving coumarin-type anticoagulants. Individuals from the medical and nursing hospital staff provided normal controls.

RESULTS

Characterization of labile serum factor. Figures 1, 2, and 3 illustrate the labile serum factor defect. This particular patient had an abnormal thromboplastin generation test when his serum was aged 24 hours at 37° C (Figure 1). Substrate plasma clotting times were all above 60 seconds, and no clot formed in the incubation tube. Serum aged only 3 hours resulted in abnormal thromboplastin generation also, although the deficiency was less. Normal serum aged 24 hours completely corrected the defect. When the patient's serum was aged only 1.5 hours, a normal test resulted.

The substitution of platelets for soybean phosphatide as the source of platelet factor 3 led to

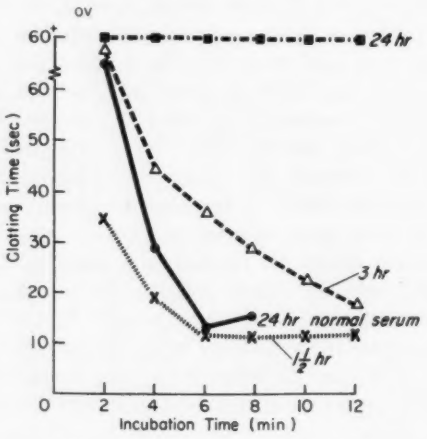

FIG. 1. THROMBOPLASTIN GENERATION IN THE LABILE SERUM FACTOR DEFICIENCY. The incubation times of the thromboplastin generation mixture are shown on the horizontal axis and the clotting times of the substrate plasma are plotted on the vertical axis. The appended "number of hours" refers to the aging of the patient's serum unless otherwise indicated.

[2] Thrombin, Topical, Parke-Davis & Co.

[3] Courtesy of Dr. John B. Graham, University of North Carolina.

[4] Courtesy of Dr. Walter H. Seegers, Wayne State University (thrombin, lot 591222; prothrombin, lot 600203).

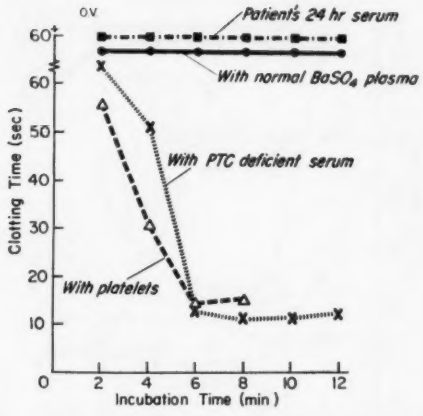

FIG. 2. CORRECTION OF THE LABILE SERUM FACTOR DEFECT BY PLATELETS AND PTC DEFICIENT SERUM.

normal thromboplastin generation even though the patient's 24-hour serum was used (Figure 2). This occurred with platelets either from the patient or from a normal subject. Serum deficient in PTC (factor IX) effected complete correction of the patient's 24-hour serum. $BaSO_4$-adsorbed normal plasma was not corrective. Stuart (factor X) deficient serum similarly restored normal thromboplastin generation (Figure 3). Normal serum adsorbed with barium sulfate did not correct the defect. These results suggested that the labile serum factor differed from PTC, PTA and the Stuart factor, was more labile on aging than these factors, and was adsorbed by barium sulfate.

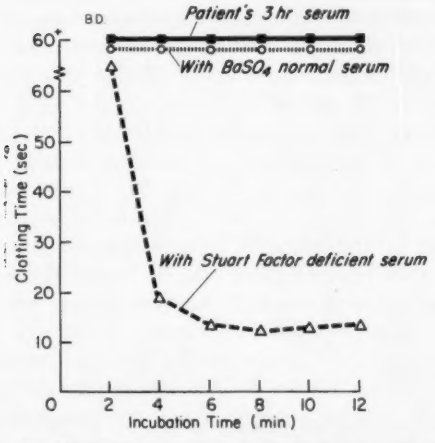

FIG. 3. CORRECTION OF THE LABILE SERUM FACTOR DEFECT BY STUART DEFICIENT SERUM.

The role of thrombin. The formation of a clot in the incubation tube in the normal thromboplastin generation test has already been mentioned. This clot appeared about the time that thromboplastin became maximal in the mixture, as indicated by a minimal clotting time of 10 to 12 seconds of the substrate test plasma. Thus, it seemed likely that the formation of thrombin and the generation of a potent thromboplastin were linked closely in the thromboplastin generation test. This suggested the possibility that normal thromboplastin generation was dependent upon the catalytic effect of a trace of thrombin in the aged serum. Perhaps the aging of the serum from some individuals resulted in the complete inactivation of the traces of thrombin.

The effects of various amounts of thrombin upon thromboplastin generation in this labile serum defect are shown in Figure 4. Even trace amounts of thrombin, as little as 0.024 U added to the incubation tube, corrected the labile serum defect. Ten to 20 times this amount of thrombin greatly accelerated thromboplastin generation; the usual lag period was eliminated. The addition of this minute amount of thrombin did not result in thromboplastin generation unless soybean phosphatide, aged serum and $BaSO_4$-adsorbed plasma were all present in the mixture. The minimal

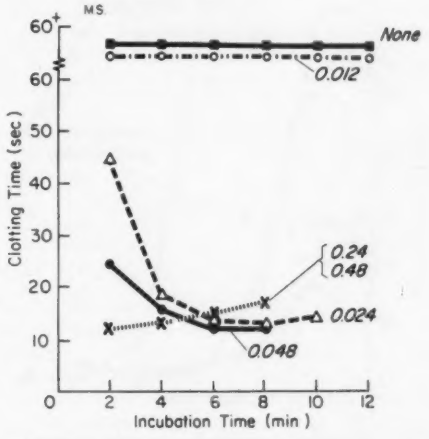

FIG. 4. THE EFFECTS OF DIFFERENT CONCENTRATIONS OF THROMBIN ON THROMBOPLASTIN GENERATION IN THE LABILE SERUM FACTOR DEFICIENCY. The patient's 3 hour old serum, normal plasma, and soybean phosphatide were used in all tests. The units of thrombin added are appended to each curve.

amount of thrombin, usually 0.024 U, which was required to correct the labile serum defect, was much too small to be detected by ordinary methods; e.g., it would not clot fibrinogen or plasma.

In most of these studies the thrombin was added to the incubation tube as a thrombin-saline diluent of the serum reagent. If it was added as a part of the saline diluent for the barium sulfate-adsorbed plasma, a similarly normal thromboplastin generation test resulted. As might be expected, however, thrombin added to the substrate plasma did not correct an abnormal thromboplastin generation test resulting from the labile serum factor defect. It was essential that the thrombin be present in the incubation mixture during the period of thromboplastin generation. This amount of thrombin, 0.024 U, had no or little effect upon the greatly impaired thromboplastin generation found in patients with hemophilia (factor VIII deficiency) and with PTC (factor IX) deficiency. Twenty-two patients with hemophilia and eight patients with PTC deficiency were so studied.

Because the thrombin reagent used (Thrombin, Topical) might be contaminated with other substances, more highly purified thrombin was tested in the thromboplastin generation system having this labile serum factor defect. The corrective property of this purified thrombin preparation was also compared with that of purified prothrombin, as shown in Figure 5. The purified thrombin corrected the defect in a manner similar to the correction obtained with Thrombin, Topical of the same strength. Purified prothrombin was also corrective but only when amounts many times larger were used. The addition of 1.3 U of prothrombin was necessary to produce the same effect obtained with 0.024 U of thrombin, either as purified thrombin or as Thrombin, Topical.

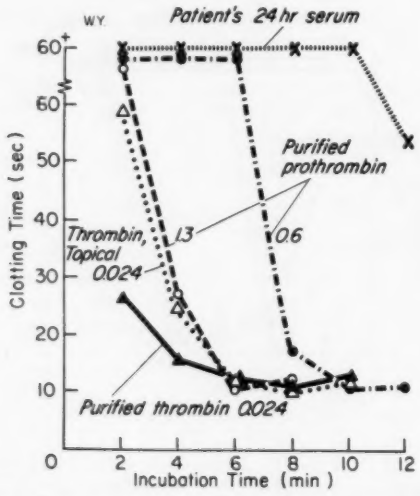

FIG. 5. EFFECTS OF PURIFIED THROMBIN, THROMBIN, TOPICAL, AND PURIFIED PROTHROMBIN ON THE THROMBOPLASTIN GENERATION IN THE LABILE SERUM FACTOR DEFICIENCY. Units per milliliter indicated for each curve.

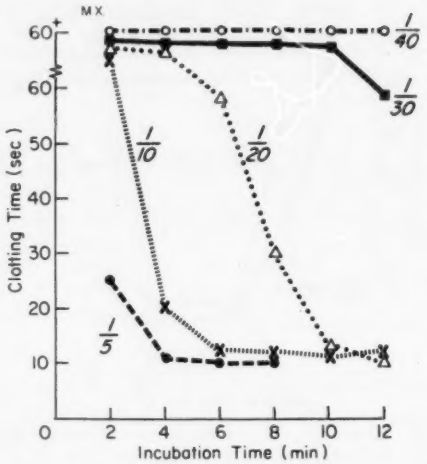

FIG. 6. THE LABILE SERUM FACTOR DEFECT IN THROMBOPLASTIN GENERATION WITH THE DILUTION OF NORMAL SERUM. The appropriate dilution of normal serum is appended to each thromboplastin generation curve.

Quantitative aspects of the labile serum factor. The labile serum factor defect has developed spontaneously with the aging of some sera from 3 to 24 hours at 37° C. A similar defect also could be produced in normal serum by dilution of a given sample. As shown in Figure 6, the yield of thromboplastin progressively declined with successive serum dilutions with saline until, at a $\frac{1}{40}$ dilution, none was produced. The critical dilution range of five normal sera so tested was between $\frac{1}{20}$ and $\frac{1}{40}$. With thromboplastin generation thus rendered abnormal by serum dilution, complete correction was obtained by the use of any one of the following (Figure 7): 0.024 U of thrombin; PTC (Factor IX) deficient serum; Stuart factor (factor X) deficient serum; and the use of platelets in place of soybean phosphatide. It seemed evident that this labile serum factor could be diluted beyond effective concentration

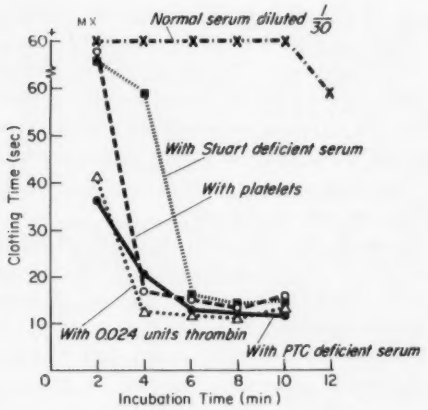

FIG. 7. THE CORRECTION OF THE LABILE SERUM FACTOR DEFECT INDUCED IN DILUTED NORMAL SERUM. Normal serum was diluted 1/30 for use in all tests.

in normal aged serum without the loss of effective amounts of PTC and the Stuart factor.

In addition, the ability of normal serum to correct the labile serum deficiency in thromboplastin generation was lost progressively with dilution. Table I-A shows the corrective effects of normal serum in varied dilutions on the labile serum defect. The normal serum was added to the serum defective in the labile serum factor in a ratio of 1 : 1 and the thromboplastin generation test performed. In this instance, also, there was a critical dilution which failed to correct the defect—i.e., at $\frac{1}{20}$.

TABLE I

Corrections of the labile serum factor defect by various dilutions of normal serum and PTC deficient serum *

Serum component 0.3 ml			Thromboplastin generation
Dilution	Amount	Amount	
	ml	*ml*	*% of normal*
A. Normal serum		Patient's serum	
		0.30	90
1/5	0.15	0.15	104
1/10	0.15	0.15	78
1/20	0.15	0.15	53
1/30	0.15	0.15	29
B. PTC deficient serum			
		0.30	30
1/30	0.15	0.15	111
1/100	0.15	0.15	84
1/1,000	0.15	0.15	0

* Normal barium sulfate-adsorbed plasma and normal substrate plasma were used. The patient's serum was diluted 1/5 for all tests. Saline was the diluent.

PTC deficient serum in serial dilutions was supplied to a test system containing serum with the labile factor defect (Table I-B). It was observed that considerably lesser amounts of PTC deficient serum than of normal serum corrected the abnormal thromboplastin generation of this patient's serum. This result implied that, whatever the corrective factor might be, it was present in higher concentration in the PTC deficient serum than in normal serum. Since PTC deficient serum has poor prothrombin conversion, prothrombin is one substance in higher concentration in PTC deficient serum than in normal serum when both are aged for 3 hours. Thrombin may also be involved, since in serum with only partial prothrombin conversion thrombin continues to form slowly until all of the prothrombin is converted.

The incubation of thrombin and platelets individually with serum. Serum with the labile factor defect had the capacity to nullify the corrective effects of 0.024 to 0.048 U of thrombin during the course of an hour's incubation of this serum with thrombin (Table II). This occurred with either Thrombin, Topical, or purified thrombin. The incubation of thrombin, alone and diluted as for use in the TGT for 1 hour at 37° C, did not de-

TABLE II

Incubation of thrombin and platelets individually with serum *

	Thromboplastin generation
	% of normal
A. Test systems: serum alone and serum plus thrombin	
Serum alone	0
Serum + 0.048 U of thrombin	
No incubation	117
Incubation for 1 hr	0
Serum + 0.048 U of thrombin†	100
B. Test systems: serum alone and serum plus platelets	
Serum alone	0
Serum plus platelets	
No incubation	111
Incubation for 1 hr	113

* The serum and thrombin, and serum and platelets were incubated 1 hour at 37° C before use in the thromboplastin generation test. The serum used had been shown deficient in the labile serum factor after being aged 24 hours at 37° C.

† Thrombin previously incubated 1 hour at 37° C.

TABLE III

Effect of heat on the labile factor in normal and PTC deficient serum *

Serum reagent				
Patient's serum (labile serum factor defect) Amount	Normal serum		Thrombin addition	Thromboplastin generation
	Preparation	Amount		
ml	*min/° C*	*ml*	*U*	*% of normal*
0.30				0
0.15	Usual	0.15		100
0.15	15/50	0.15		57
0.15	30/50	0.15		44
0.15	60/50	0.15		28
0.15	5/56	0.15		0
0.15	15/50	0.15	0.024	102
0.15	60/50	0.15	0.024	94
0.15	5/56	0.15	0.024	54
	PTC deficient serum			
0.15	60/50	0.15		100
0.15	5/56	0.15		20

* Normal serum was heated for different periods of time at various temperatures. It was then used to correct the labile serum factor defect. PTC deficient serum was similarly tested. The other reagents in the thromboplastin generation test were as previously described. The patient's serum and the normal serum were diluted 1/5 with saline before use in the quantities indicated.

stroy its ability to correct the labile serum defect.

Prothrombin (1.3 U) then was incubated with the defective serum. Such a mixture retained its original ability to provide normal thromboplastin generation. Of course, this amount of prothrombin was very large as compared with the amount of thrombin used.

The corrective action of platelets on the TGT was not destroyed after an hour's incubation of the serum and platelets together. The antithrombin of the serum deficient in the labile factor was similar to that of normal serum.

Heat stability of the labile serum factor. The heat stability of this factor in normal and PTC deficient serum is given in Table III. These sera tended to lose their ability to correct the labile serum defect with progressive heating. For example, normal serum heated 15 minutes at 50° C only partially corrected the abnormal thromboplastin generation of the labile serum defect. Further heating at 50° C provided additional losses of the labile factor in normal serum. The heating of the normal serum to 56° C for 5 minutes led to complete loss of activity. The addition of 0.024 U of thrombin completely restored the capacity of the normal serum heated to 50° C to generate thromboplastin. Thrombin did not correct completely normal serum heated to 56° C. Heating was less effective in destroying the corrective capacity of PTC deficient serum, perhaps because of the larger amounts of residual prothrombin and thrombin present in this serum.

Patients and subjects with the labile serum factor defect. With all of these individuals, the defect was manifest in serum aged for 3 hours. Thirty-three patients with different types of clinical bleeding of unknown cause had abnormal thromboplastin generation because of the labile serum factor defect. These included patients with gastrointestinal bleeding, nosebleeds, easy bruising, operative and postoperative bleeding, and subdural hematomas. In none of these patients were there found local lesions or other hematological and coagulative abnormalities to explain the bleeding. The labile serum factor defect continued to be present when the same patients were restudied on subsequent occasions. Some patients had required transfusions. Four others, however, had had abdominal surgery performed without excessive operative or postoperative bleeding.

Many of these patients had a family history of bleeding. The labile serum factor defect was found in two sisters who bruised easily and bled excessively after dental extractions. It was demonstrated in a mother and daughter, both with a history of bleeding. It was found in the son and two grandsons of a man who had died of unexplained massive gastrointestinal bleeding.

The labile serum factor defect was present in 10 of 13 patients with polycythemia vera. In addition to elevated hemoglobin, erythrocyte and hematocrit values, most of these patients had elevated platelet counts. Their thromboplastin generation defect was severe and typically was corrected by serum aged 1 or 2 hours, thrombin 0.024 U, PTC deficient serum, or platelets from normal subjects.

Ten other patients with abnormal thromboplastin generation due to deficient labile serum factor included three patients with hereditary hemorrhagic telangiectasia, two with thrombasthenia and five with myelofibrosis.

Umbilical cord blood was obtained from new-

born infants. The sera obtained from these blood specimens were used in the thromboplastin generation test. The barium sulfate-adsorbed plasma and the substrate plasma used were from normal adults. Nine of the ten newborn sera tested had abnormal thromboplastin generation, characteristic of the labile serum factor defect. In these, the test results ranged from 0 to 74 per cent, with five sera producing no thromboplastin generation.

One hundred two studies were performed in 75 "normal" adult subjects. In only six of these was the thromboplastin generation abnormal when the serum was aged 3 hours. In each instance the abnormality was caused by the typical labile serum defect which tended to be moderate rather than severe. The test results were 71, 70, 60, 43, 41 and 20 per cent for these six tests.

Labile serum factor defect in liver disease. The thromboplastin generation test was abnormal in 33 of 47 patients with hepatic cirrhosis. With serum aged for 3 hours, thromboplastin generation ranged from 0 to 74 per cent. In 28 of these patients the serum defect fitted well into the labile serum factor classification in that it was corrected by the addition of 0.024 U of thrombin, by the patient's serum aged only 1 or 2 hours, by normal aged serum, usually by the use of platelets, by PTC deficient serum, or by Stuart deficient serum (this latter attempted in only one instance). Eleven of these patients were studied subsequently on 20 occasions and continued to demonstrate the labile serum factor defect. Three patients did not have the defect when restudied later but had had clinical improvement of the liver disease. The five other patients with abnormal thromboplastin generation had multiple defects which included Stuart factor and PTC as well as the labile serum factor. Their tests were only partially corrected by serum aged 1 hour, by platelets, by 0.024 U of thrombin, or by PTC deficient serum.

Most of the patients with cirrhosis had associated reduced prothrombin and factor VII concentrations; some had, in addition, increased fibrinolytic activity. The 14 other patients with normal thromboplastin generation had low prothrombin and factor VII concentrations.

The labile serum factor defect and coumarin drugs. Thromboplastin generation in patients receiving coumarin therapy was almost always abnormal. This abnormality, as is described elsewhere, was caused usually by Stuart factor and PTC deficiencies (9). In some patients, however, the labile serum factor defect contributed somewhat to the thromboplastin deficiency. Figure 8 illustrates this point. Serum aged only 1 hour, the addition of thrombin, or the use of platelets all corrected partially the very abnormal thromboplastin generation caused by defects in the patient's 3-hour serum.

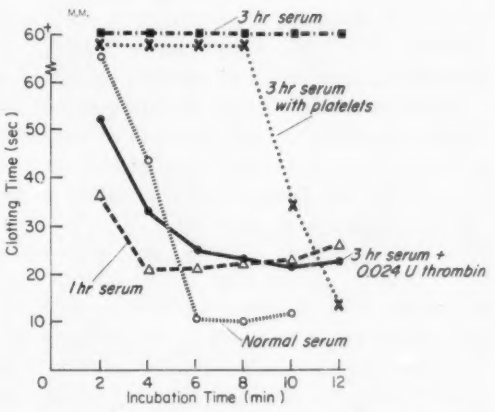

FIG. 8. THE LABILE SERUM FACTOR DEFECT IN A PATIENT RECEIVING DICUMAROL. His prothrombin time by the one-stage test was 22.5 seconds (control 13.6 seconds).

Cross correction studies. On many occasions serum from one patient with the labile serum factor defect was used in an attempt to correct the abnormal thromboplastin generation test of another patient with a similarly identified defect. In no instance was the slightest correction obtained. A failure of correction also resulted when sera from the patients with liver disease or from those receiving coumarin drugs were so tested.

DISCUSSION

The "labile serum factor" deficiency in thromboplastin generation encountered in these patients had some of the attributes of a PTC deficiency. Normal serum provided complete correction; the failure of normal barium sulfate-adsorbed plasma to correct the abnormal thromboplastin generation test ruled out deficiencies of factor VIII, factor V, PTA and the "glass" factor. When it was found that PTC deficient serum completely corrected the defect, however, it was clear that a PTC deficiency was not the explanation. In a similar

manner, the possibility of a Stuart factor deficiency was ruled out. The next question arose as to what other known serum factor could be defective.

Serum, of course, contains initially many of the substances present in plasma and, in addition, the substances formed in the process of clotting. In 1904 Bordet and Gengou called attention to the clot-promoting activity of serum (10). They believed that this activity in serum might be thrombin or a derivative. Serum from normal subjects aged for 3 hours at 37° C does not exhibit thrombic activity, however, as indicated by an ability to clot fibrinogen or plasma. It might still contain traces of thrombin which might catalyze thromboplastin generation and other reactions.

Others have shown that serum contains clot-promoting activity apparently different from thrombin (11, 12); DeVries, Alexander and Goldstein have termed such activity SPCA or serum prothrombin conversion accelerator (11). It is now most commonly recognized as factor VII. Ware and Seegers have also stressed the presence of serum accelerator globulin (factor VI) in serum (13). PTC (factor IX), the Stuart factor (factor X) and PTA are other well recognized components of aged serum.

Factor VII has been considered unnecessary for the thromboplastin generation test (14). It is also much more stable than the "labile serum factor," since it resists heating to 56° C for 30 minutes (14). The plasma from patients with the "labile serum factor" defect had a normal factor VII level. Such information suggests that the labile serum factor was probably not factor VII.

In 1955 Duckert, Flückiger, Matter and Koller described a serum factor, necessary for thromboplastin formation, which was absent in sera from Dicumarol-treated patients, the newborn, and patients with hepatitis and cirrhosis (3). Their serum factor was adsorbed by barium sulfate and disappeared from normal sera in a few hours to a few days when stored at room temperature. The thromboplastin generation test in the patients with this defect became much worse when they used a brain extract as a source of platelet factor 3 instead of platelets. Recently Koller, of this same group, has indicated that the serum factor which they described was primarily the Stuart factor (factor X) (15). Spaet and Kropatkin produced a thromboplastin generation deficiency in normal sera aged in the refrigerator for 1 week or longer (4). It was distinguished from Stuart factor or prothrombin deficiencies but was thought to be closely related to prothrombin or a prothrombin derivative. This defect was also found in liver disease (16). Abnormal thromboplastin generation from a serum defect resulted when normal serum was incubated with thorium (17). The thorium-induced deficiency was corrected by PTC (factor IX) and PTA deficient sera but not by refrigerator-aged serum or Dicumarol serum.

The labile serum factor which we have described appears somewhat similar to the factors originally described by Duckert and co-workers and by Spaet and Kropatkin (3, 4). Our results have indicated that a deficiency of this factor was produced readily in sera from certain patients by aging 3 to 24 hours at 37° C. It was destroyed in normal sera by incubation at 50° C. A defect of this factor could be produced quantitatively in normal serum by serial dilutions with saline. The factor was essential for normal thromboplastin generation with the use of soybean phosphatide as the source of platelet factor 3.

A trace amount of purified thrombin (0.024 U) corrected the labile serum factor defect. This amount, insufficient to clot fibrinogen or plasma, was adequate to restore normal thromboplastin generation to sera with the defect. Thrombin in this small quantity did not effect normal thromboplastin generation in PTC or in AHG deficiencies. Purified prothrombin, in much larger and measurable amounts, also restored normal thromboplastin generation.

Serum with the labile factor defect had the capacity to nullify the usual corrective effect of thrombin if the thrombin and serum were incubated together prior to use in the TGT. Such serum destroyed or inactivated this small amount of thrombin. The corrective effect of platelets was not destroyed by incubation with the serum.

Platelets are known to possess a number of factors with blood clotting activity (14). Of these, platelet factor 3 is the only one known to be essential for thromboplastin generation. Our results suggest that platelets also contain another essential factor which is also present in normal aged serum. Desforges and Bigelow have shown that platelets previously incubated with thrombin have greatly enhanced thromboplastic activity

(18). Perhaps such activity is attributable to adsorption of thrombin to platelets. The ability of platelets to correct the labile serum factor defect may then depend upon their adsorption of a trace of thrombin or like substance.

These studies of the labile serum factor defect suggest that thrombin in minute amounts must be present for normal thromboplastin generation. That thrombin has such a catalytic role in the TGT is not surprising in view of its profound effect upon so many other blood clotting tests. For example, thrombin activates antihemophilic globulin (19); it converts accelerator globulin into an active form (13); it aids in the conversion of prothrombin to thrombin (20); it "labilizes" platelets (21). We have no information about the mechanism through which thrombin may exert its effect in thromboplastin generation.

Biggs, Douglas, and Macfarlane found that a low concentration of thrombin, too small to clot plasma but which would clot fibrinogen in about 150 seconds, accelerated normal thromboplastin generation (22). Miale and Wilson suggested that the thrombic activity observed to develop in the incubation tube of the TGT might have importance in thromboplastin formation (23). They found that the addition of 0.25 U of thrombin accelerated normal thromboplastin formation and reduced the usual lag period. This amount was much greater than the 0.024 U needed to correct the labile serum defect.

We do not know that the labile serum factor defect has any relationship to hemorrhagic disease. Since it develops only with the aging of serum, perhaps it is only a laboratory curiosity. Yet this defect was encountered in many patients with clinical bleeding otherwise unexplained and in some other patients with other known coagulation defects. The *in vitro* ability of a serum to destroy this labile serum factor or traces of thrombin, as the case may be, may bear a relationship to such an *in vivo* phenomenon as a bleeding tendency.

The thromboplastin generation test has a growing use in clinical laboratories for the diagnosis of hemorrhagic disease. Should the platelet factor 3 reagent be supplied as a soybean or brain phosphatide instead of as a platelet suspension, it is clear that an abnormal TGT will result from time to time from the labile serum factor defect. Such an abnormal TGT must be interpreted properly after the performance of detailed corrective studies. Only thus can the over-diagnosis of a disease like PTC (factor IX) deficiency be avoided.

SUMMARY AND CONCLUSIONS

A labile serum factor necessary for thromboplastin generation has been described. A deficiency of this factor appears in the serum of some patients which has been aged 3 to 24 hours. The defect manifests itself when soybean phosphatide is used as the source of platelet factor 3 in the thromboplastin generation test. Studies to date suggest that the labile factor is a trace of thrombin and that, in sera showing the defect, such traces of thrombin are destroyed in a few hours. If so, this is simply another illustration of the importance of thrombin as a catalyst for clotting reactions.

We have found the labile serum factor defect in many patients with otherwise unexplained bleeding of clinical significance. In addition, it has consistently been detected in the newborn and in patients with liver disease, with polycythemia vera, and after coumarin drugs. It was rarely found in normal adult individuals whose serum was aged for 3 hours at 37° C. Whether or not this serum abnormality has real importance in the production of hemorrhagic disease remains to be established.

The labile serum factor defect can be confused readily with deficiencies of other serum factors, especially with PTC (factor IX). Such confusion seems especially likely in the quantitative studies with the thromboplastin generation test since this factor, probably thrombin, can readily be diluted beyond effective concentration in aged normal serum.

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QUANTITATIVE STUDIES OF NASAL CARRIERS OF STAPHYLOCOCCI AMONG HOSPITALIZED PATIENTS *

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Several investigators have shown that patients who are nasal carriers of staphylococci have a higher incidence of postoperative infections and other types of staphylococcal disease than have patients who are not nasal carriers (1, 2). In these studies, the increased incidence of infections was due predominantly to staphylococci of the same phage type as those isolated from the nose of the patient before the infections occurred.

If the nasal carrier is a major source of his own hospital-acquired staphylococcal infection, qualitative and quantitative changes in the nasal flora of staphylococci should be important in understanding the origin of a portion of staphylococcal disease. Several reports have been made of qualitative studies of nasal carriers of staphylococci in hospitalized patients (3–6). Williams and colleagues (1) found that carriers of tetracycline-resistant strains had a higher incidence of infections than had carriers of tetracycline-sensitive strains. Also carriers of "virulent" strains, which were defined as staphylococci causing two or more infections in a short period, had more frequent infections than carriers of "nonvirulent" strains.

Quantitative studies of nasal carriers have been reported from a mental hospital having a reservoir of largely drug-susceptible staphylococci (7, 8). Similar studies have been continued in a general hospital with a large proportion of physically ill patients and a large reservoir of antibiotic-resistant staphylococci. In this setting, the dissemination of staphylococci to a patient's environment was correlated with the quantity of staphylococci that could be recovered from his nose. In addition, carriers of 80/81 staphylococci and patients with certain diseases were found to be heavier nasal carriers and therefore potentially greater disseminators of staphylococci than were patients without these characteristics.

MATERIALS AND METHODS OF STUDY

Nasal cultures were made 3 to 5 times per week on all patients on a male medical ward, a female medical ward or a male surgical ward at the Louisville General Hospital in the period from October 1958 to August 1959.

During this period sweep plates were made of the patients' clothing by sweeping open 100×15 mm petri dishes with Tryptic Soy Agar (Difco) over the anterior surface of the patients' gowns from shoulder to groin three times on each side.

The laboratory procedures employed have been described in detail previously (7) but some of the pertinent procedures are summarized below.

Both sides of the nose were swabbed with sterile cotton applicators moistened in broth. The swabs were shaken in a measured volume of broth, and coagulase-positive pigmented colonies were enumerated in appropriate dilutions. In the present report the term staphylococci is restricted to coagulase-positive *micrococcus pyogenes* var. *aureus*.

Phage typing was performed using the following 22 phages: group I, 29, 52, 52a, 79, 80; group II, 3a, 3b, 3c, 55, 71; group III, 6, 7, 42e, 47, 53, 54, 73, 75, 77, Va_1; and unclassified, 81, 42b.

Phages were propagated in heart infusion broth with 0.004 mole per L of added $CaCl_2$ (9). The staphylococci were first tested with the lowest concentration of phage which lysed the propagating strain and, if fewer than 20 plaques were present with all phages, they were then retested with a 1,000-fold higher concentration. Staphylococci which reacted with more than one phage were classified according to the predominant reactions.

Drug susceptibility tests were performed by inoculating the surface of an agar plate into which specified concentrations of various antibiotics were incorporated with an overnight culture of the strain to be tested. Staphylococci were considered susceptible to penicillin if they were inhibited by 1.6 μg per ml and to tetracycline, chloramphenicol, erythromycin, kanamycin, novobiocin or furaltadone if inhibited by 6.2 μg per ml of these drugs. Organisms not inhibited by these concentrations were considered to be resistant.

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† John and Mary R. Markle Scholar in Medical Science.

TABLE I

Susceptibility of staphylococci from hospital-acquired lesions or from nasal carriers to penicillin, tetracycline, erythromycin and chloramphenicol

Resistant to:	Lesions, 100 strains		Carriers, 2,134 strains	
	%		%	
None of the 4 drugs	9.0		24.6	
Pen. only	10.0		21.1	
Pen. and tetra.	29.0		18.1	
Pen., tetra. and erythro.	10.0		8.0	
Pen., tetra. and chlor.	8.0	79.0*	5.1	44.6*
Pen., tetra., chlor. and erythro.	32.0		13.4	
Other resistance patterns	2.0		9.7	
Total	100.0		100.0	

* Strains from lesions were more frequently resistant to both penicillin and tetracycline than were strains from carriers (χ^2:$p < 0.01$).

TABLE II

Phage patterns of staphylococci from hospital-acquired lesions or from hospitalized nasal carriers

Phage reactions	Lesions, 100 strains	Carriers, 2,134 strains
	%	%
Group I	4.0	2.3
Group II	11.0	7.6
Group III	8.0	30.4
Not typeable	13.0	23.1
Misc.	0.0	10.5
80/81	64.0*	26.1*
Total	100.0	100.0

* More strains from lesions than strains from carriers were lysed by phages 80/81 (χ^2:$p < 0.01$).

RESULTS

Drug susceptibility of staphylococci from hospital-acquired lesions and from nasal carriers. In Table I are shown the major resistance patterns of 100 strains of staphylococci isolated from hospital-acquired lesions and of 2,134 strains isolated from nasal carriers.

Fifty-four of the strains from lesions were isolated from patients who had received antimicrobial drugs before the onset of staphylococcal infections. In 31 patients, antibiotics were administered to prevent postoperative or other infections and in 23 patients, for the treatment of other bacterial diseases including pneumococcal pneumonia, pyelonephritis and bacteremias. Thirty-four patients received penicillin with or without streptomycin, 10 received chloramphenicol, 2 tetracycline, 4 penicillin and erythromycin, and 4 patients received both penicillin and chloramphenicol.

Staphylococci isolated from lesions were more frequently resistant to each of the four drugs and to both penicillin and tetracycline than were

TABLE III

Antimicrobial resistance of staphylococci of various phage groups

Phage group	Resistant to: Pen. only	Pen., tetra.	Pen., tetra., eryth.	Pen., tetra., chlor.	Pen., tetra., chlor., eryth.	Total multiple resistance
	%	%	%	%	%	%
Group I 50 strains	24.0	0.0	6.0	0.0	4.0	10.0
Group II 160 strains	34.4	3.1	0.0	1.3	1.3	5.7
Not typeable 493 strains	16.9	7.1	6.3	6.7	13.8	34.9
Misc. 225 strains	43.5	12.4	4.4	2.7	2.2	21.7
Group III 649 strains	26.4	13.6	13.2	4.3	12.6	43.7*
80/81 557 strains	5.2	41.3	7.2	7.0	22.8	78.3*

* Strains lysed by phages 80/81 were more frequently multiple drug-resistant than were strains lysed by phages of group III (χ^2:$p < 0.01$).

staphylococci isolated from carriers. In both groups, staphylococci resistant to the more recently introduced antibiotics tended also to be resistant to the older drugs; thus, 95.2 per cent of staphylococci resistant to tetracycline were also resistant to penicillin, 97.0 per cent of staphylococci resistant to erythromycin were resistant to both penicillin and tetracycline, and 74.3 per cent of staphylococci resistant to chloramphenicol were also resistant to both penicillin and tetracycline. Novobiocin, kanamycin and furaltadone (Altafur) have been used infrequently in this hospital and less than 1 per cent of staphylococci was resistant to any of the three drugs.

Phage types of staphylococci from hospital-acquired lesions and from nasal carriers. When the staphylococci from lesions and carriers were phage-typed it was found that 64 per cent of the staphylococci from lesions were susceptible to phages 80 and/or 81, but only 26 per cent of staphylococci from carriers were lysed by these phages (Table II). Thirty per cent of strains from carriers were lysed by phages belonging to group III, but group III staphylococci were responsible for only 8 per cent of hospital-acquired lesions.

Drug susceptibility of staphylococci of various phage groups. There was a marked difference in the frequency with which staphylococci of various phage groups were multiple drug-resistant. Seventy-eight per cent of phage type 80/81 staphylococci was resistant to both penicillin and tetracycline and 37 per cent was resistant to both of these drugs and to erythromycin or chloramphenicol or both (Table III). Forty-four per cent of phage group III staphylococci were resistant to both penicillin and tetraycline and 30 per cent were resistant to both of these drugs and also to erythromycin or chloramphenicol or both. A much smaller proportion of staphylococci in other phage groups was multiple drug-resistant.

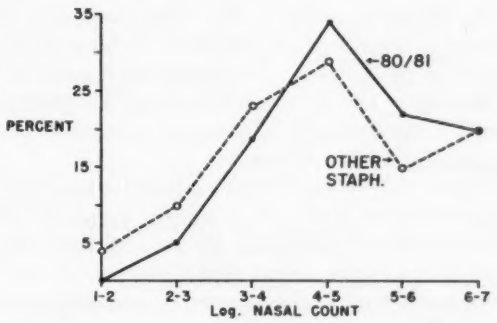

FIG. 1. CULTURES FROM UNTREATED CARRIERS OF THE 80/81 STRAIN CONTAINED 10,000 OR MORE COLONIES PER SWAB MORE FREQUENTLY THAN DID CULTURES FROM UNTREATED CARRIERS OF OTHER TYPES OF STAPHYLOCOCCI.

Correlation of phage types and quantitative nasal cultures. Quantitative nasal cultures were obtained from 175 patients without overt staphylococcal infections who did not receive systemic antibiotics before the cultures were taken. Seventy-seven per cent of 82 cultures from carriers of 80/81 staphylococci contained more than 10^4 colonies per swab, but only 64 per cent of 420 cultures from carriers of other types of staphylococci contained this many colonies (Figure 1).

Correlations between carrier rates and quantitative counts. When at least five consecutive nasal cultures were made from 106 patients who

TABLE IV

Quantitative nasal cultures in untreated patients with different carrier rates

Carrier rate	Patients	Cultures	Positive cultures	Quantitative counts of positive cultures			
				$>10^3$	$>10^4$	$>10^5$	$>10^6$
	No.	*No.*	*No.*	*%*	*%*	*%*	*%*
0	41	348	0				
1–20	15	168	21	79.2	31.6*	5.3	5.3
20–40	5	82	18	88.9	61.1	16.7	0.0
40–60	11	144	70	82.3	63.2	22.0	10.3
60–80	6	63	44	90.9	75.0	38.6	15.9
80–100	28	333	292	89.6	73.6*	47.2	27.2
Total	106	1,138	445				

* Patients with carrier rates of 80 to 100 per cent had a larger proportion of positive cultures with more than 10^4 colonies per swab than had patients with carrier rates of 1 to 20 per cent (χ^2: $p < 0.01$).

did not receive antimicrobial drugs, staphylococci were isolated from 28 patients in at least 80 per cent of the cultures. No staphylococci were isolated from any of the cultures from 41 patients, and in the remaining 37 patients, the carrier rates ranged from 0 to 80 per cent.

Only 5.3 per cent of the positive cultures from patients with carrier rates between 1 and 20 per cent contained more than 10^5 colonies per swab. In cultures from patients with higher carrier rates, the proportion of positive cultures containing more than 10^5 per swab progressively increased so that 47.2 per cent of positive cultures from patients with carrier rates of 80 to 100 per cent contained at least this number (Table IV). Similarly, 31.6 per cent of positive cultures from patients with carrier rates of 1 to 20 per cent, and 73.6 per cent of positive cultures from patients with carrier rates greater than 80 per cent, contained more than 10^4 colonies per swab. Thus, patients with the highest carrier rates also tended to have the greatest number of staphylococci in the positive cultures.

Relationship between quantitative counts and dissemination of staphylococci. An attempt was made to correlate nasal counts with dissemination of staphylococci by making quantitative nasal counts and sweep plates of the clothing of patients without staphylococcal infections, approximately 4 hours after their bedclothes had been changed. Staphylococci were isolated from 43 per cent of 80 sweep plates from patients with nasal counts greater than 10^4 colonies per swab, but from only 24 per cent of 37 plates from patients with counts less than 10^4 colonies per swab (Figure 2). Staphylococci isolated from plates were the same phage type as staphylococci in the patient's nose

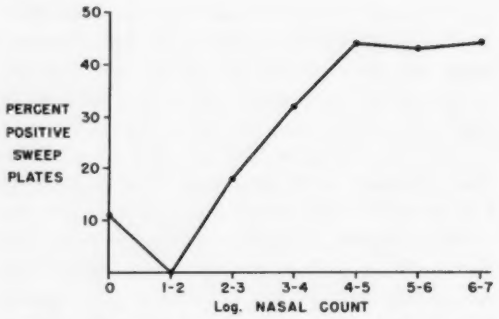

FIG. 2. SWEEP PLATES OF PATIENTS' CLOTHING CONTAINED COAGULASE-POSITIVE STAPHYLOCOCCI IN OVER 40 PER CENT OF SAMPLES FROM CARRIERS OF 10,000 OR MORE STAPHYLOCOCCI PER SWAB. Coagulase-positive staphylococci were isolated less frequently from the clothing of noncarriers or carriers of fewer than 10,000 colonies per swab.

in all but 6 of 44 positive sweep plates from carriers. In the remaining 6 patients and in the 20 positive sweep plates from noncarriers, staphylococci were presumably obtained from sources other than the nose of the patient.

The effect of antimicrobial drugs on nasal cultures. There was no significant difference in the carrier rates of patients receiving penicillin, as compared with patients who did not receive antimicrobial drugs (Table V). In patients receiving erythromycin, to which only 21 per cent of all strains from carriers were resistant, the carrier rate fell from 44 per cent during the first three days of treatment to 18 per cent in patients who received erythromycin for longer than 9 days. The carrier rate was higher in patients treated with tetracycline than in untreated patients in all periods of treatment.

TABLE V

Carrier rates in patients receiving antimicrobial drugs

Treatment No. of days	Penicillin, 76 patients		Tetracycline, 23 patients		Erythromycin, 41 patients		No treatment, 271 patients	
	Cultures	Carrier rate	Cultures	Carrier rate	Cultures	Carrier rate	Cultures	Carrier rate
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
1–3	76	31.6	25	48.0	59	44.1	295	30.2
4–6	63	27.0	21	57.1	52	19.2	251	26.7
7–9	53	32.1	16	50.0	36	30.6	245	31.4
>9	94	52.1	22	63.6	48	18.8	713	45.3
Total	286	37.2	84	54.8*	195	29.3	1,504	37.0

* Patients treated with tetracycline had a higher carrier rate and patients treated with erythromycin a lower carrier rate than untreated patients (χ^2:$p<0.01$).

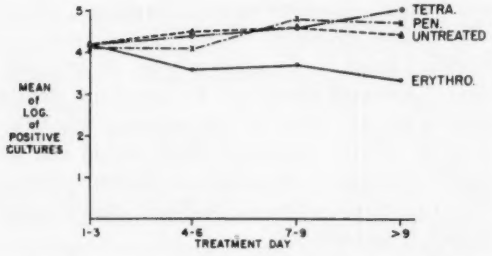

FIG. 3. ONLY TREATMENT WITH ERYTHROMYCIN LOWERED THE MEAN NUMBER OF STAPHYLOCOCCI ISOLATED FROM CARRIERS; TREATMENT WITH PENICILLIN OR TETRACYCLINE DID NOT CHANGE THE NUMBER OF STAPHYLOCOCCI ISOLATED.

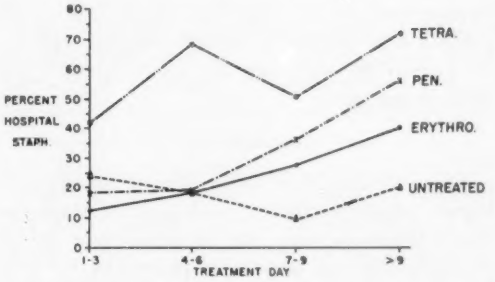

FIG. 4. THE PROPORTION OF STAPHYLOCOCCI WHICH WERE RESISTANT TO BOTH PENICILLIN AND TETRACYLINE AND LYSED BY PHAGES 80/81 OR PHAGES OF GROUP III INCREASED DURING TREATMENT WITH ALL THREE ANTIMICROBIAL DRUGS. No significant changes occurred in untreated patients.

The number of staphylococci recovered from patients who remained carriers did not change during treatment with penicillin or tetracycline, as compared with untreated patients (Figure 3). During treatment with erythromycin there was a significant decrease in the number of staphylococci.

In addition to changes in carrier rates and in quantitative counts, treatment with all three drugs increased the proportion of staphylococci which were resistant to penicillin and tetracycline and lysed by either phages 80/81 or by phages of group III (Figure 4).

Quantitative counts in untreated patients with various illnesses. The distribution of counts from 106 untreated patients with at least five consecutive cultures was not a bell-shaped curve but was skewed to counts greater than 10^6. It was found that the positive cultures from patients with diabetes, patients with illnesses which terminated in death during hospitalization, and patients with staphylococcal infections contained more than 10^4 colonies per swab more frequently than did positive cultures from untreated patients with other diseases (Table VI).

No significant differences could be detected in the distribution of counts from patients with other illnesses, but only a few patients with leukemias or disseminated carcinomas were studied.

In the 26 untreated patients with staphylococcal infections, nasal cultures were negative for

TABLE VI

Quantitative counts in untreated patients with different illnesses

	Diabetics	Fatal illnesses	Staph. infections	Other patients (with more than 5 cultures)
No. of patients	21	15	26	91
No. of cultures	94	65	26	1,020
Carrier rates	45.7	44.6	84.6*	36.0
	Quantitative counts of positive cultures			
	%	%	%	%
10^1–10^2				2.2
10^2–10^3				10.4
10^3–10^4	4.7			21.5
10^4–10^5	34.9	27.6	22.7	32.4
10^5–10^6	30.2 } 95.3*	31.0 } 100.0*	9.1 } 100.0*	15.8 } 65.9*
10^6–10^7	30.2	41.4	68.2	17.7
Total	100.0	100.0	100.0	100.0

* Patients with diabetes, fatal illnesses or staphylococcal infections had a larger proportion of positive cultures with more than 10^4 colonies per swab (χ^2: $p < 0.1$), and the carrier rate in patients with staphylococcal infections was higher than in all other untreated patients.

staphylococci in only 4, and in all but 3 of the 22 patients with positive nasal cultures the staphylococci from the nose and the lesion were of the same phage type.

DISCUSSION

These studies have shown that a significantly higher proportion of hospital-acquired staphylococcal infections was due to phage type 80/81 staphylococci than could be explained by the proportion of these staphylococci in the nose of hospitalized patients. In part, the predominance of this organism might be explained by the observation that staphylococci lysed by 80/81 phages were more frequently multiple drug-resistant than were staphylococci of other phage groups. Most of the infections occurred in patients receiving antibiotics for other reasons, and drug-resistant strains would tend to be selected as the etiological agents. In this way, 80/81 staphylococci would be more frequently selected than strains which were less frequently drug-resistant.

In addition, carriers of phage type 80/81 staphylococci tended to have larger numbers of staphylococci in the nose than had carriers of other staphylococci, in the absence of antimicrobial therapy. Gould (10) has demonstrated that considerable amounts of penicillin can be recovered from the noses of hospital patients who are not receiving systemic penicillin; aerial dissemination in areas of mixing and administration of the drug was thought to be responsible for this finding in his studies. By this means, it is possible that penicillin could depress the quantity of penicillin-susceptible strains that could be recovered and would provide an explanation for the increased numbers of staphylococci observed in carriers of 80/81 strains which were usually penicillin-resistant. Since contamination of a patient's environment was more frequent in patients with higher counts, 80/81 strains would tend to be more widely disseminated because of their increased quantity in carriers.

In several reports, an increased virulence of phage type 80/81 staphylococci has been postulated to explain the frequency with which this strain can be isolated from hospital-acquired lesions (11, 12). Elek and Conen (13) have reported that this strain does not produce lesions more frequently than other strains when injected into human volunteers in equal numbers; therefore, by this criterion, no evidence of increased virulence could be assigned to type 80 staphylococci. Although there may be significant differences in the capacity of various strains of staphylococci to initiate infections which would not be manifest in such experiments, no direct evidence for an increased virulence of 80/81 staphylococci in man is available.

If virulence is not a factor, the reports that carriers of this strain have a higher incidence of infection than have other nasal carriers when they are removed from the hospital environment (11, 12) suggest that even in the absence of intensive antimicrobial therapy, larger numbers of staphylococci are available from the nose of carriers of 80/81 staphylococci than from other carriers. No comparisons have been reported of the number of staphylococci of different phage types than can be recovered from individuals outside of hospitals, but some consideration should be given to the number of bacteria to which an individual is exposed in order to evaluate the virulence of a particular strain.

Although this report is concerned primarily with the relationship between nasal carriers and staphylococcal infections, it is probable that infected cases serve as major sources of further dissemination of staphylococci, once established. In the series reported by Williams and co-workers (1) the incidence of infections in patients who were not nasal carriers was 2 per cent, but 15 per cent of patients who were nasal carriers of tetracycline-resistant strains became infected. The increased incidence of infections was due to staphylococci of the same phage type as those present in the patient's nose before the infection appeared. If these figures are generally true, the staphylococci present in the patient's nose represent a greater direct hazard to the patient than do the staphylococci from lesions of other patients. Furthermore, the argument that a particular strain predominates in hospital infections because the infected case is a major source of infection, and this source was infected with this particular strain, does not satisfactorily explain why a few strains of the many different ones available from carriers should be a major cause of disease throughout the country (14).

Several studies have shown that patients may

be intermittent or relatively constant nasal carriers of coagulase-positive staphylococci (15, 16). As shown in these studies, patients with high carrier rates also have a larger number of staphylococci in positive cultures than have patients with lower carrier rates. At the present time there is insufficient evidence to indicate that only consistent carriers are important in staphylococcal infections, but with additional data it may be possible to limit programs, designed to control staphylocca! infections through control of carriers, to consistent carriers of large numbers of staphylococci.

The effect of antimicrobial drugs on carrier rates and quantitative counts seemed to be related to the availability of drug-resistant staphylococci. Thus, tetracycline and penicillin to which 45 and 66 per cent of the strains were resistant, did not decrease either the carrier rates or the quantitative counts in patients who remained carriers. Erythromycin, to which only 21 per cent of the strains was resistant, reduced both carrier rates and quantitative counts in carriers. The changes observed during erythromycin treatment in this study were less marked than those observed in a mental hospital where no strains were resistant to erythromycin (7).

All three antimicrobial drugs increased the proportion of staphylococci which were multiple drug-resistant and lysed by phages of group III or phages 80/81. Staphylococci with these characteristics are responsible for a large majority of hospital-acquired infections throughout the country (14) and, as shown in these and other studies, antimicrobial therapy is responsible for maintaining a large reservoir of these organisms in nasal carriers within hospitals.

The possible explanation for the higher carrier rates and higher quantitative counts in patients with diabetes, staphylococcal infections and fatal illnesses is even more complex. Three of the 22 carriers with staphylococcal infections were observed to have nasal counts of greater than one million colonies per swab for two to three months before the appearance of staphylococcal skin infections. In each instance, the staphylococci from the nose and from the lesions were of the same phage type. No cultures were taken from the remaining patients until staphylococcal infections were present and, therefore, it was not possible to determine whether high nasal counts preceded or followed staphylococcal infections. Since large numbers of organisms were present in the nose, they constituted a reservoir from which staphylococci could be disseminated, in addition to the obvious hazards of an open lesion.

Patients with diabetes or debilitating diseases also have a higher incidence of staphylococcal infections (17). The large numbers of nasal staphylococci in patients with these diseases may be a reflection of a generalized increased susceptibility to infections due to unknown causes.

Regardless of the explanations, the presence of large numbers of nasal staphylococci in patients may be important in the dissemination of staphylococci to the hospital environment and in the etiology of the patient's own hospital-acquired staphylococcal infection.

SUMMARY

A larger proportion of hospital-acquired staphylococcal infections was due to phage type 80/81 staphylococci than could be explained by the prevalence of these staphylococci in the noses of hospitalized patients. In part, these observations may be explained by the findings that staphylococci lysed by 80/81 phages were more frequently multiple drug-resistant than other staphylococci and were present in larger numbers in uninfected, untreated nasal carriers than were other strains.

Sweep plates of patients' clothing were more frequently positive for coagulase-positive staphylococci in patients with nasal counts greater than 10,000 colonies per swab than in patients with counts below 10,000.

Higher nasal quantitative counts of positive cultures were observed in untreated patients with high carrier rates and in patients with diabetes, staphylococcal infections or fatal illnesses.

The predominant effect of antimicrobial drugs on the carrier status was to select multiple-resistant strains. The effects of drugs on carrier rates and quantitative counts were related to the availability of staphylococci resistant to the administered drug.

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GENERAL AND REGIONAL CIRCULATORY RESPONSES TO CHANGE IN BLOOD pH AND CARBON DIOXIDE TENSION *

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The pH and the carbon dioxide tension of the blood flowing through a tissue are commonly thought to be major factors in the local control of regional blood flow, for instance in the vasodilation in exercising muscle (1). They also are of recognized importance in the control of general circulatory homeostasis; for example, systemic hypertension accompanies acutely induced hypercapnia (2, 3), and hypotension the hypocapnia of hyperventilation (4). Furthermore, disorders of acid-base equilibrium occur in pathologic states with sufficient frequency to merit consideration of the role of blood pH and CO_2 tension in control of the circulation burdened by disease such as the acidosis of uremia and diabetic coma or the alkalosis of vomiting.

With these considerations in mind, the present studies were undertaken in healthy humans to determine the effects of variation in blood pH and CO_2 tension (P_{CO_2}) on cardiac output and on the flow of blood through extremity musculature. In an attempt to separate direct effects of change in pH or P_{CO_2} on blood vessels themselves from those mediated by the nervous system, sympathetic impulses to the extremity were blocked with phenoxybenzamine (Dibenzyline [1]) administered intra-arterially to the extremity under study.

METHODS

The subjects studied were healthy male volunteers and one male with essential hypertension. Their ages ranged from 22 to 46 years. Most had participated repeatedly in similar studies in the same laboratory and were, therefore, relaxed and at ease.

Acidemia was induced by intravenous infusion of 500 to 800 ml of 0.2 M lactic acid or 0.17 M ammonium chloride, and alkalosis by infusion of 500 to 600 ml of 0.5 M sodium bicarbonate. The duration of the NH_4Cl or lactic acid infusions ranged from 3 to 4 hours. Bicarbonate infusion duration varied from 45 minutes to 2.5 hours. Experiments in which arterial pH changed less than 0.05 U were excluded from the results. Normal saline, 500 to 600 ml, was administered to control subjects to observe the effects of infusion of similar volumes without pH change. In 8 subjects, 5 to 10 ml of 0.5 M $NaHCO_3$ (pH measured as 8.8) was infused directly into a brachial artery. In the same subjects similar volumes of normal saline or of 0.5 M NaCl were injected intra-arterially as control determinations.

Hypercapnia was induced by inhalation of 5 or 7 per cent carbon dioxide in air for periods of 5 to 15 minutes, and hypocapnia by vigorous voluntary hyperventilation of room air. In experiments designed to ascertain the changes in cardiac output induced by vigorous respiratory movements without change in arterial P_{CO_2}, the CO_2 concentration of expired air was continuously monitored with a Liston-Becker infrared CO_2 analyzer. During voluntary hyperventilation, carbon dioxide was added to inspired air at a rate adjusted to keep end-expiratory CO_2 concentration constant at the level present during normal breathing. As will be shown, use of this technic enabled maintenance of nearly constant arterial CO_2 tension during both quiet breathing and vigorous hyperventilation.

Forearm or calf blood flow was estimated by venous-occlusion plethysmography as described by Wise (5), with plethysmograph temperature 34° C (6), using a Statham P97 low-pressure strain gage as transducer and a Sanborn Polyviso recorder. The control and experimental estimates of extremity blood flow used for every subject were each the average of 10 or more consecutive individual flow determinations made at 30-second intervals.

Cardiac output was estimated by the indicator-dilution technic of Stewart (7) and Hamilton, Moore, Kinsman and Spurling (8). Evans blue dye, 40 mg, or indocyanine green dye, 10 mg, was injected into an antecubital vein and immediately followed by 20 ml of saline to propel the bolus of dye rapidly into the central venous circulation. Intermittent samples of blood were collected from a brachial artery at 2-second intervals through a no. 15 needle and a 5 inch piece of PE 240 polyethylene tubing. Dye concentrations in successive plasma samples were measured in a Coleman Junior spectrophotometer. Calculations were made as described by Doyle, Wilson, Lépine and Warren (9).

Sympathetic blockade of the forearm was induced by

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[1] Supplied through the courtesy of Dr. E. J. Fellows, Smith, Kline & French Laboratories.

injection of phenoxybenzamine into the brachial artery in the upper third of the bicipital groove. Phenoxybenzamine was injected at a rate of 1 to 2 mg in 1 minute. Injections were repeated until maximal increase in forearm flow occurred. Maximal flow was attained in 10 to 15 minutes, averaged about 3 times the control value, and was maintained as long as any recordings were made—about 4 hours. The total dose of phenoxybenzamine ranged from 6 to 12 mg, a dose which did not produce any change in blood pressure, respiratory rate or heart rate, or any subjective symptoms. Effectiveness of adrenergic blockade was suggested by observations in 8 subjects that vasocontriction produced in the intact forearm by intra-arterial epinephrine and norepinephrine was absent when these substances were injected intra-arterially after phenoxybenzamine injection.

Blood pressure and pulse rate were recorded from an intra-arterial needle with Statham strain gages. The pH of arterial blood was measured with a Cambridge model R pH meter with appropriate corrections to body temperature (10). Arterial carbon dioxide content was measured by the manometric technic of Peters and Van Slyke (11), and P_{CO_2} was estimated directly by the method of Riley, Proemmel and Franke (12) or calculated from pH, CO_2 content and hematocrit by means of the nomogram of Singer and Hastings (13). In experiments on hyperventilation and CO_2 inhalation, the volume of expired air was measured, using a Tissot spirometer.

Results were evaluated statistically by calculating the standard error of the difference between control and experimental means. The 0.95 confidence limits include the difference between means ± 2.04 times the standard error of the difference, and indicate the limits in which the difference between the means of the control and experimental groups will fall 95 times out of 100 (14). Thus, when the confidence limits do not overlap zero, there is high likelihood that the difference between means is significantly different from zero. The p value expresses the probability that the control and experimental means are not significantly different, and was calculated using the Fisher t test (14).

RESULTS

Acid and base infusions

Changes in blood gases and pH. Following both acid and base infusions, mean pH was significantly altered ($p < 0.01$) from the control value (Tables I, II and III). Sodium bicarbonate infusions were accompanied by a significant increase in arterial P_{CO_2} and CO_2 content in all experiments. Acid infusions were associated with moderate reduction in CO_2 content and insignificant changes in CO_2 tension.

General circulatory effects. As shown in Table I, alkalosis was accompanied by a 32 per cent increase in mean cardiac output ($p < 0.01$). Individual values for cardiac index (Figure 1) rose following $NaHCO_3$ infusion in 9 of 12 determinations and were unchanged in 3. Average total peripheral resistance decreased 22 per cent ($p < 0.05$) following bicarbonate infusion. In general, larger pH changes were associated with greater increases in cardiac output. With induced acidosis (Table I), no significant change in mean cardiac output occurred. Total peripheral resistance likewise was unchanged following reduction in blood pH. Figure 1 demonstrates the changes in cardiac index observed in individual subjects following acid and $NaHCO_3$ infusions. Infusions of 500 to 600 ml of 0.16 M NaCl produced no significant change in arterial pH or in cardiac output in any of five individuals. Heart rate and mean arterial pressure showed little change following acid or base infusions. Depth of respiration usually increased after acid infusion, but respiratory rate was not changed. Sodium bicarbonate infusions did not noticeably affect respiratory rate or depth.

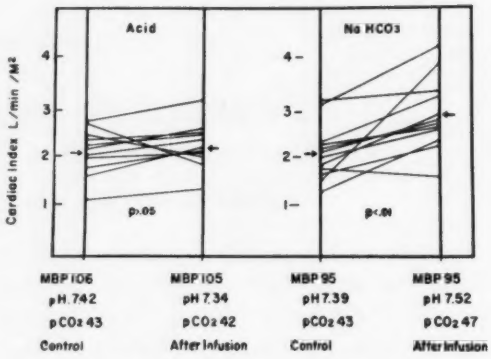

FIG. 1. CHANGE IN CARDIAC INDEX ASSOCIATED WITH INFUSIONS OF $NaHCO_3$ OR OF NH_4Cl OR LACTIC ACID. Arrows point to the mean values in each group; MPB = mean arterial blood pressure.

Changes in extremity flow. Neither induced acidosis nor alkalosis produced significant change in blood flow in the intact forearm (Table II). Average values for forearm flow were identical before and after $NaHCO_3$ infusion despite significant ($p < 0.01$) change in arterial pH. Following acid infusion, average forearm flow increased, but the change was not statistically significant. Forearm flow increased in four of six experiments in which pH was significantly reduced by acid in-

TABLE I

Effects of change in blood pH on general circulation

		Control mean, N = 12	Experimental mean,* N = 12	Difference between means	0.95 Confidence limit	p
A. $NaHCO_3$ infusion						
Arterial pH	Units	7.39	7.52	+ 0.13	+0.10– + 0.16	<0.01
Art. P_{CO_2}	mm Hg	43.1	48.4	+ 5.3	+3.1 – + 7.5	<0.01
Art. CO_2 content	mmoles/L	22.8	33.4	+10.6	+7.8 – +13.4	<0.01
Cardiac index	L/min/m²	3.8	5.0	+ 1.2	+0.6 – + 1.8	<0.01
Peripheral resistance	mm Hg/ L/min	13.8	10.7	− 3.1	−5.2 – − 1.0	<0.05
Heart rate		78.1	84.3	+ 6.2	+1.0 – +11.4	<0.05
B. Acid infusion		N = 10	N = 10			
Arterial pH	units	7.42	7.34	− 0.08	−0.10– − 0.06	<0.01
Art. P_{CO_2}	mm Hg	41.7	39.8	− 1.9	−7.2 – + 3.4	>0.1
Art. CO_2 content	mmoles/L	23.6	18.7	− 4.9	−8.0 – − 1.8	<0.01
Cardiac index	L/min/m²	3.6	3.8	+ 0.2	−0.4 – + 0.8	>0.1
Peripheral resistance	mm Hg/ L/min	16.0	14.9	− 1.1	−3.8 – + 1.6	>0.1
Heart rate		77.0	73.0	− 4.0	−8.9 – + 0.9	>0.1

* During intravenous infusion of 0.5 M $NaHCO_3$, 0.2 M lactic acid, or 0.17 M NH_4Cl, see Methods.

TABLE II

Effects of change in blood pH on blood flow in intact forearm

		Control mean, N = 6	Experimental mean,* N = 6	Difference between means	0.95 Confidence limit	p
A. $NaHCO_3$ infusion						
Arterial pH	units	7.40	7.49	+0.09	+0.08– +0.10	<0.01
Art. P_{CO_2}	mm Hg	40.7	45.0	+4.3	+2.0 – +6.6	<0.05
Art. CO_2 content	mmoles/L	20.8	26.0	+5.2	+2.6 – +7.8	<0.01
Forearm flow	ml/100 g/ min	4.4	4.4	+0.01	−0.34– +0.36	>0.1
Forearm resistance	mm Hg/ ml/100 g/ min	23.5	25.5	+2.0	−0.7 – +4.7	>0.1
B. Acid infusion		N = 6	N = 6			
Arterial pH	units	7.42	7.35	−0.07	−0.12– −0.02	<0.05
Art. P_{CO_2}	mm Hg	38.0	42.7	+4.7	−0.4 – +9.8	>0.1
Art. CO_2 content	mmoles	20.5	19.8	−0.7	−3.4 – +2.0	>0.1
Forearm flow	ml/100 g/ min	4.4	5.9	+1.5	−0.1 – +3.1	>0.1
Forearm resistance	mm Hg/ ml/100 g/ min	22.0	17.7	−4.3	−9.4 – +0.8	>0.1

* During intravenous infusion of 0.5 M $NaHCO_3$, 0.2 M lactic acid, or 0.17 M NH_4Cl; see Methods.

TABLE III

Effects of change in blood pH on blood flow in forearm after phenoxybenzamine

		Control mean, N = 8	Experimental mean,* N = 8	Difference between means	0.95 Confidence limits	p
A. $NaHCO_3$ infusion						
Arterial pH	units	7.39	7.54	+ 0.15	+0.10– + 0.20	<0.01
Art. P_{CO_2}	mm Hg	40.6	49.9	+ 9.3	+6.8 – +11.8	<0.01
Art. CO_2 content	mmoles/L	21.4	34.0	+12.6	+9.8 – +15.4	<0.01
Forearm flow	ml/100 g/ min	13.5	19.8	+ 6.3	+2.9 – + 9.7	<0.01
Forearm resistance	mm Hg/ ml/100 g/ min	7.5	5.1	– 2.4	–4.5 – – 0.3	<0.05
B. Acid infusion		N = 8	N = 8			
Arterial pH	units	7.42	7.33	– 0.09	–0.06– – 0.12	<0.01
Art. P_{CO_2}	mm Hg	42.9	38.5	– 4.4	–9.2 – + 0.4	>0.1
Art. CO_2 content	mmoles/L	23.3	17.8	– 5.5	–7.4 – – 3.6	<0.01
Forearm flow	ml/100 g/ min	13.8	17.9	+ 4.1	+0.4 – + 7.8	<0.05
Forearm resistance	mm Hg/ ml/100 g/ min	10.0	8.5	– 1.5	–0.5 – – 2.5	<0.05

* During intravenous infusion of $NaHCO_3$ or NH_4Cl, following intra-arterial administration of phenoxybenzamine (Dibenzyline).

fusion, was unchanged in one, and decreased slightly in the sixth.

Following blockade of sympathetic impulses to the forearm with intra-arterial phenoxybenzamine, significant increases in blood flow occurred as arterial pH was shifted in either direction from control values (Table III, Figure 2). Increase in pH associated with intravenous $NaHCO_3$ infusion was accompanied by a 47 per cent increase ($p < 0.01$) in mean forearm flow with individual increases ranging from 15 to 160 per cent. Decreased arterial pH following NH_4Cl infusions was associated with a 28 per cent ($p < 0.05$) increase in average forearm flow. Individual values increased in six of eight experiments with NH_4Cl and decreased slightly in two. Since blood pressure was unchanged, forearm resistance decreased significantly following acid and base infusions.

When 0.5 M $NaHCO_3$ was injected directly into the brachial artery, large (three- to sevenfold) increases in forearm flow occurred in each of eight subjects studied. Intra-arterial injection of similar volumes of normal saline or hypertonic (0.5 M) NaCl produced no change in forearm flow in the same eight subjects.

Other effects. Infusions of sodium bicarbonate produced no symptoms. Ammonium chloride infusions were accompanied by nausea and vomiting and by local pain at the site of injection if the

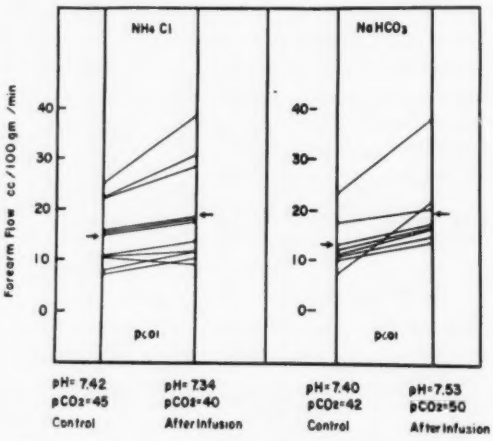

FIG. 2. CHANGES, ASSOCIATED WITH INTRAVENOUS NH_4Cl OR $NaHCO_3$ ADMINISTRATION, IN BLOOD FLOW THROUGH THE FOREARM DEPRIVED OF ITS SYMPATHETIC INNERVATION BY PREVIOUS INTRA-ARTERIAL INFUSION OF PHENOXYBENZAMINE (DIBENZYLINE). Arrows indicate mean values.

TABLE IV

Effects of hypercapnia on blood flow in the intact extremity

		Control mean, N = 11	Experimental mean,* N = 11	Difference between means	0.95 Confidence limit	p
Arterial pH	units	7.39	7.31	− 0.08	−0.10– − 0.06	<0.01
Art. P_{CO_2}	mm Hg	40.4	52.5	+12.1	+ 8.8– +15.4	<0.01
Art. CO_2 content	mmoles/L	20.6	23.5	+ 2.9	+ 1.9– + 3.9	<0.01
Limb blood flow	ml/100 ml/min	7.0	7.9	+ 0.9	−0.2 – + 2.0	>0.05
Limb. vasc. resistance	mm Hg/ ml/100 ml/min	14.2	14.0	− 0.2	−2.4 – + 2.0	>0.05
Heart rate	beats/min	87.5	95.3	+ 7.8	+2.3 – +13.3	<0.05

* Five or 7 per cent CO_2, breathed for 5 to 10 minutes.

rate of infusion exceeded 350 ml per hour. Lactic acid infusions produced pain at the site of venipuncture if the rate of administration exceeded 250 ml per hour. No systemic symptoms were noted following lactic acid infusion, but in one subject (F. S.) hemoglobinuria developed immediately following the infusion and persisted for several hours. No oliguria or azotemia resulted. Tenderness and induration of the vein into which lactic acid had been infused, with erythema of the overlying skin, developed in three of five subjects given lactic acid.

Effects of CO_2 inhalation and hyperventilation on extremity blood flow

Changes in blood gases and pH. Following CO_2 inhalation there was marked elevation in arterial P_{CO_2} and reduction in pH (Tables IV and V). With the subjects breathing room air, the arterial P_{CO_2} averaged 40 mm Hg, whereas during CO_2 inhalation arterial P_{CO_2} rose to 56 mm Hg, a significant increase of 35 per cent. Arterial pH fell from a mean of 7.38 while the patients were breathing room air to 7.30 during inhalation of CO_2, this change being statistically significant. Similar magnitudes of change in pH, P_{CO_2}, and CO_2 content occurred in both the control and phenoxybenzamine groups. Inhalation of 7 per cent CO_2 raised average arterial P_{CO_2} more (average increase in P_{CO_2}, 17 mm Hg) than did 5 per cent CO_2 (average rise 12 mm Hg), but there was considerable individual variability in response of arterial P_{CO_2} to these two concentrations of inspired gas.

During voluntary hyperventilation, changes in pH and P_{CO_2} were achieved which were slightly

TABLE V

Effects of hypercapnia on extremity after phenoxylbenzamine

		Control mean, N = 8	Experimental mean,* N = 8	Difference between means	0.95 Confidence limit	p
Arterial pH	units	7.38	7.29	− 0.09	− 0.13– − 0.05	<0.01
Art. P_{CO_2}	mm Hg	40.1	54.9	+14.8	+10.9 – +18.7	<0.01
Art. CO_2 content	mmoles/L	20.3	23.3	+ 3.0	+ 1.4 – + 4.0	<0.01
Limb blood flow	ml/100 ml/min	14.4	23.7	+ 9.3	+ 3.7 – +14.9	<0.02
Limb vasc. resistance	mm Hg/ ml/100 ml/min	6.4	4.6	− 1.8	− 2.7 – − 0.9	<0.01
Heart rate	beats/min	94.1	112.9	+18.8	+ 9.2 – +28.4	<0.01

* Five to 7 per cent CO_2, breathed for 5 to 10 minutes.

TABLE VI

Effects of voluntary hyperventilation on blood flow in intact forearm

		Control mean, N = 11	Experimental mean,* N = 11	Difference between means	0.95 Confidence limits	p
Arterial pH	units	7.39	7.57	+ 0.18	+ 0.15– + 0.21	<0.01
Art. P_{CO_2}	mm Hg	42.0	25.6	−16.4	−17.6 – −15.2	<0.01
Art. CO_2 content	mmoles/L	21.5	19.0	− 2.5	− 4.9 – − 0.1	<0.10
Blood flow	ml/100 ml/min	4.2	5.1	+ 0.9	+ 0.3 – + 1.5	<0.05
Vascular resistance	mm Hg/ ml/100 ml/min	24.1	20.2	− 3.9	− 7.3 – − 0.5	<0.05
Heart rate	beats/min	77.2	88.8	+11.6	+ 6.0 – +17.2	<0.01
Ventilation	L/min	7.8	36.5	+28.7	+19.6 – +37.8	<0.01

* Vigorous voluntary hyperventilation for 5 to 7 minutes.

greater in magnitude and, of course, opposite in direction to those occurring during induced hypercapnia, as shown in Table VI. Arterial pH rose from an average of 7.40 during normal breathing to 7.54 during hyperventilation, while CO_2 tension fell from a control average of 42 to 28 mm Hg, accompanying two- to sixfold increases in ventilation. Correlation between increase in ventilation and fall in arterial P_{CO_2} was fair ($R = 0.5$, $p < 0.05$).

Changes in extremity blood flow. In the intact extremity, hypercapnia produced slight and inconsistent changes in blood flow despite marked change in P_{CO_2} and pH (Table I). Figure 3 shows similar inconsistent changes in extremity resistance accompanying hypercapnia. After phenoxybenzamine, comparable elevation in arterial P_{CO_2} was accompanied by large increase in extremity flow (40 per cent; $p < 0.02$) and decrease in extremity vascular resistance. As demonstrated in Figure 3, extremity resistance decreased 29 per cent ($p < 0.01$). Individual changes in blood flow correlated well with degree of reduction in pH (Figure 4).

Hypocapnia was accompanied by moderate increases in blood flow in the intact forearm (Table

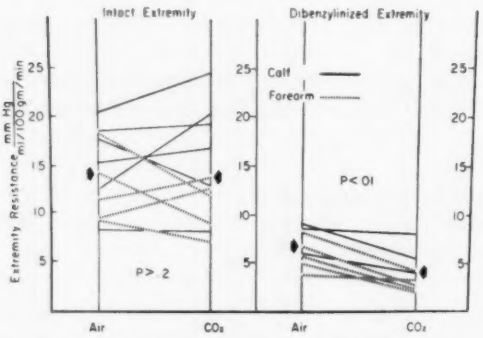

FIG. 3. INHALATION OF 5 OR 7 PER CENT CARBON DIOXIDE PRODUCED NO CHANGE IN RESISTANCE TO BLOOD FLOW IN THE INTACT EXTREMITY BUT WAS ACCOMPANIED BY 29 PER CENT DECREASE IN VASCULAR RESISTANCE IN THE EXTREMITY DEPRIVED OF SYMPATHETIC STIMULI BY INTRA-ARTERIAL INJECTION OF PHENOXYBENZAMINE. Arrows point to the mean of each vertical volume. The horizontal lines connect determinations of resistance in the same subject before and during CO_2 inhalation.

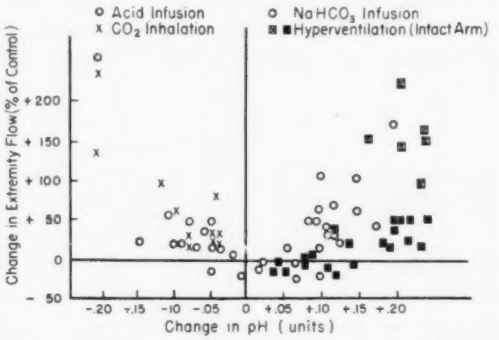

FIG. 4. RELATION BETWEEN CHANGE IN pH AND ALTERATION IN EXTREMITY BLOOD FLOW. All data refer to the extremity following phenoxybenzamine injection except the data regarding voluntary hyperventilation, which denote changes in the intact arm. Boxed crosses represent data of Burnum, Hickam and McIntosh (4); see Discussion.

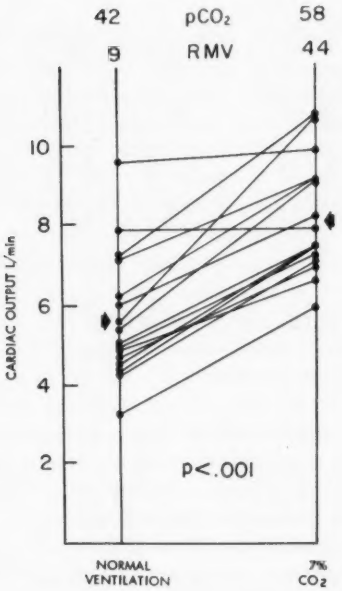

FIG. 5. THE EFFECT OF INHALATION OF 7 PER CENT CARBON DIOXIDE ON CARDIAC OUTPUT. RMV = respiratory minute volume in liters per minute, and P_{CO_2} = arterial CO_2 tension in millimeters of mercury.

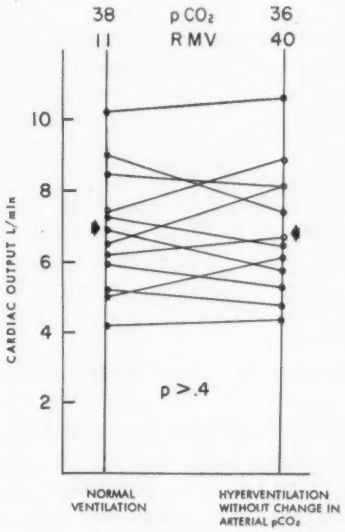

FIG. 6. THE EFFECT ON CARDIAC OUTPUT OF VIGOROUS VOLUNTARY HYPERVENTILATION DURING WHICH ARTERIAL CO_2 TENSION WAS MAINTAINED AT LEVELS PRESENT DURING NORMAL BREATHING. Abbreviations as in Figure 5.

VI). Average flow increased 20 per cent ($p < 0.05$), and individual changes in forearm flow correlated reasonably well with changes in pH, as shown in Figure 4.

In these experiments, blood pressure and heart rate rose significantly ($p < 0.01$) during CO_2 inhalation (Table IV and V), blood pressure increasing in 17 of 18 individual experiments and heart rate in 15 of 18. Hypocapnia, on the other hand, produced no significant change in blood pressure (see Table VI) or in heart rate, despite changes in P_{CO_2} of comparable magnitude. Average blood pressure was 8 mm Hg lower ($0.05 < p < 0.02$) and heart rate was slightly higher after phenoxybenzamine administration, during both air and CO_2 breathing.

Effects of CO_2 inhalation on the general circulation

The circulatory effects of breathing 7 per cent CO_2 are shown in Figure 5 and Table VII. Accompanying the rise in respiratory minute volume from 9 to 44 L per minute and in arterial CO_2

TABLE VII
Circulatory effects of induced hypercapnia

		Control mean, N = 16	Experimental mean,* N = 16	Difference between means	0.95 Confidence limits	p
Arterial pH	units	7.38	7.25	− 0.13	− 0.11 − − 0.15	<0.01
Art. P_{CO_2}	mm Hg	42.0	58.5	+16.5	+13.3 − +19.7	<0.01
Cardiac index	L/min/m²	2.9	4.2	+ 1.3	+ 1.0 − + 1.6	<0.01
Peripheral resistance	mm Hg/ L/min	16.8	13.1	− 3.7	− 2.3 − − 5.1	<0.01
Heart rate	beats/min	70.6	88.3	+17.7	+12.4 − +23.0	<0.01
Ventilation	L/min	9	44	+34.9	+27.5 − +42.3	<0.01

* Seven per cent CO_2, breathed for 7 minutes.

TABLE VIII

Effect of hyperventilation without change in arterial CO_2 tension on the circulation

		Control mean, N = 12	Experimental mean,* N = 12	Difference between means	0.95 Confidence limits	p
Arterial pH	units	7.37	7.38	+ 0.01	− 0.01 − + 0.03	>0.1
Art. P_{CO_2}	mm Hg	37.7	36.1	− 1.6	− 4.7 − + 1.5	>0.1
Cardiac index	L/min/m²	3.6	3.6	0	− 0.4 − + 0.4	>0.1
Peripheral resistance	mm Hg/ L/min	14.2	15.0	+ 0.8	− 0.6 − + 2.2	>0.1
Heart rate	beats/min	69.5	74.7	+ 5.2	+ 1.1 − + 9.3	<0.05
Ventilation	L/min	11.8	39.6	+27.8	+21.9 − +33.7	<0.01

* Vigorous voluntary hyperventilation of CO_2 in low concentration; see Methods.

tension from 42 to 52 mm Hg during the CO_2 inhalation, cardiac output rose in every subject and attained a mean of 8.2 L per minute, a value 45 per cent greater than the control mean of 5.7 L per minute. The mean arterial pressure of the group rose from an average of 89 to 105 mm Hg, and heart rate increased from 70 to 87 beats per minute. Stroke volume increased by 20 per cent, while calculated total peripheral resistance fell by 23 per cent. Changes in cardiac output, blood pressure, heart rate, stroke volume and peripheral resistance were significant at the $p < 0.01$ level.

When two control observations were performed, before and after the experimental observations in 12 experiments, good reproducibility of the cardiac output measurement was demonstrated. Cardiac output averaged 6.2 L per minute in the first and 5.9 L per minute in the second control determinations.

Similar degrees of hyperventilation, with maintenance of a constant arterial CO_2 tension, produced insignificant changes in circulatory dynamics (Figure 6 and Table VIII). Despite increase in respiratory minute volume from 14 to 38 L per minute, cardiac output did not change significantly from its control value of 6.9 L per minute during quiet breathing. Blood pressure, heart rate, and the derived measurements of stroke volume and peripheral resistance also remained unchanged.

DISCUSSION

I. Acid and bicarbonate infusions

A. Extremity circulation. In the forearm, pretreated with intra-arterial phenoxybenzamine, blood flow increased during change of pH in either direction from control values, suggesting that such change in the pH of perfusing blood exerts a direct vasodilator effect in this region. In the intact forearm this dilation probably is prevented by competitive sympathetic vasoconstrictor impulses, initiated by pH change in blood perfusing some other area, perhaps the carotid and aortic chemoreceptors (15).

Deal and Green (16) have shown in dogs that if arteries directly supplying extremity muscle are infused with acid or alkaline solutions, there is an associated increase in muscle blood flow. Similarly, our experiments show large increases in forearm flow following infusion of 0.5 M $NaHCO_3$ directly into the brachial artery, but not after similar intra-arterial infusions of 0.5 M NaCl. It is therefore evident that change in pH of the blood immediately supplying extremity musculature results in increased flow through the extremity.

In man, the effects of change in pH of blood perfusing the carotid and aortic chemoreceptors and the vasomotor center have not been studied for obvious technical reasons. In the dog, perfusion of both carotid bodies with blood acidified by lactic acid (2 ml of 0.1 N lactic acid added to 6 ml of blood) was followed by vasoconstriction in the circulation supplied by the axillary artery, and this vasoconstriction was abolished by sympathectomy. Perfusion of the carotid bodies by blood to which 0.12 M $NaHCO_3$ had been added resulted in little change in foreleg blood flow (17). To the extent to which these findings in the anesthetized dog can be applied to the intact human, they lend credence to the view presented above

that the local vasodilating effect of reduction in pH of perfusing blood is opposed in the intact forearm by sympathetic nervous impulses originating in the carotid chemoreceptors. Similarly, the most probable explanation for absence of vasodilation in the intact extremity in response to intravenous $NaHCO_3$ infusion is an opposing neurogenic vasoconstrictor mechanism, since infusion of $NaHCO_3$ directly into the artery produces vasodilation. Since the chemoreceptors of the dog fail to respond to intracarotid $NaHCO_3$ infusion by inducing extremity vasoconstriction (17), it is necessary to invoke species difference in chemoreceptor responsiveness to explain the results observed in man, or to postulate another mechanism —possibly baroreceptor-induced vasoconstriction in response to a tendency to lowered blood pressure accompanying the decrease in total peripheral resistance observed during bicarbonate infusion.

B. General circulatory effects. Increase in cardiac output following $NaHCO_3$ infusions, with little change in cardiac output after acid infusions, may be explained by the assumption that both reduction and elevation in blood pH exert a direct vasodilator effect on a large portion of the total vascular bed. In the case of acid infusion a competitive influence is introduced by central chemoreceptors to cause concomitant stimulation of vasoconstrictor centers in the central nervous system. On the other hand, as shown by Bernthal (17), addition of $NaHCO_3$ to blood perfusing the carotid chemoreceptor produces little stimulation of the medullary vasomotor center. The direct vasodilation produced by alkalosis is therefore unopposed by sympathetic vasoconstrictor impulses, and can bring about reduction in total peripheral resistance and secondary increase in cardiac output.

Since bicarbonate infusion produced increase in cardiac output without change in blood flow through the intact forearm, a question is raised as to the possible vascular beds to which the increased cardiac output is distributed. Increase in pH produced by bicarbonate infusion increases cerebral blood flow and decreases cerebrovascular resistance (18). Data relative to other areas of the circulation are not available, so that the question cannot be satisfactorily answered at present.

It should be noted that ammonium chloride infusions generally required a longer time for their completion than did the bicarbonate infusions because of the nausea produced by rapid NH_4Cl infusion. Furthermore, changes in pH were generally less marked in the group of acid experiments than in the bicarbonate studies. In addition, the toxic potentialities of NH_4Cl prevented infusion of equimolar concentrations of acid and $NaHCO_3$ solutions. The observed differences in response of cardiac output to acid as opposed to $NaHCO_3$ infusions may be attributable, at least in part, to these differences in experimental conditions.

II. Hypercapnia and hypocapnia

A. Extremity circulation. The fact that hypercapnia dilates extremity blood vessels after phenoxybenzamine administration, but does not modify blood flow in the intact extremity, leads to the conclusion that hypercapnia acts directly to dilate calf and forearm vasculature, and that this direct vasodilation is antagonized in the intact organism by sympathetic vasoconstrictor impulses which also result from hypercapnia, presumably from its effect on the central nervous system. In conditions in which hypercapnia occurs throughout the body, competition exists between local direct vasodilator and sympathetic vasoconstrictor effects of high CO_2. These results suggest the existence of a mechanism whereby local tissue needs for vasodilation can take precedence when there is but slight change in the general blood level of CO_2—e.g., in reactive hyperemia. On the other hand, this mechanism allows general body needs for circulatory homeostasis to have preference over local needs; for example, in the maintenance of blood pressure during general hypercapnia.

Figure 4 demonstrates information available from two sources regarding the effects of hypocapnia accompanying voluntary hyperventilation on the extremity circulation. The data of Burnum, Hickam and McIntosh (4) (shown as boxed crosses) demonstrate much larger increases in forearm blood flow for a given increase in pH than do our data. The responses to hypocapnia of their subjects differ from ours in a number of other respects, outstanding among which are the 25 per cent fall in blood pressure and the 75 per cent rise in heart rate which occurred during hypocapnia in their subjects who hyperventilated maximally

for 1 minute. Arterial P_{CO_2} fell 22 mm Hg in their group, an average decrease somewhat greater than the 14 mm Hg achieved in our patients. The more marked circulatory changes observed by Burnum and co-workers are presumably associated with the greater and more abrupt decrease in CO_2 tension induced. Despite these quantitative differences, it seems reasonable to conclude that respiratory alkalosis dilates forearm vessels, especially when it is rapidly induced and of marked degree. Available information does not permit decision as to whether this vasodilation is a direct vascular effect of hypocapnia or the result of decrease in sympathetic "tone," but the former is suggested by observations of Burnum and associates that similar increase in blood flow occurred during hypocapnia in extremities previously subjected to sympathectomy or regional nerve block.

From the data presented, it is difficult to separate the effects of pH change on extremity vasculature from those of change in CO_2 tension. Hypercapnia increases blood flow in the extremity following sympathetic blockade no matter whether pH is increased, as by $NaHCO_3$ infusion, or decreased as during 7 per cent CO_2 inhalation. Hypocapnia during voluntary hyperventilation is accompanied by vasodilation (see Figure 4) in the intact and sympathectomized forearm (4). Hypocapnia associated with primary excess of hydrogen ions dilates vessels in the "Dibenzylinized" extremity, as shown above. With regard to the intact extremity, during definite acidemia resulting from NH_4Cl infusion, there was no change in blood flow, although in our experiments, as in those of others (18), CO_2 tension was not definitely lowered during the acid infusion. It is therefore possible that vasodilation might occur in the intact extremity if arterial CO_2 pressure and pH were both reduced. These observations suggest that change in the caliber of extremity vasculature accompanies body-wide shift in acid-base balance only when fall in CO_2 tension and rise in pH occur together, namely in respiratory alkalosis, and that local change in blood pH or P_{CO_2}, in either direction from the values normally present, results in vasodilation in the extremity. The latter conclusion rests on the assumption that changes in blood flow in the extremity after phenoxybenzamine represent modifications in vascular caliber effected by the direct vascular actions of CO_2 and hydrogen ions.

In areas of the circulation other than the extremity, rapid elevation of arterial CO_2 tension produces varied effects. Cerebral vessels dilate during hypercapnia (19, 20). Sympathetic control of cerebral blood vessels is thought to be minimal (21), and in their response to hypercapnia, the cerebral vessels behave as do forearm vessels deprived of sympathetic stimuli. In their vasoconstrictive response to hypocapnia (19), on the other hand, cerebral vessels react differently from the vessels in the sympathectomized human extremity (4). Renal vasoconstriction accompanies hypercapnia in normal man, 13 per cent reduction in renal blood flow and 46 per cent increase in renal vascular resistance occurring with inhalation of 10 per cent CO_2 (22). Thus, the renal circulation reacts differently from either the cerebral or the extremity vessels. The effects of hypercapnia on splanchnic and hepatic circulation in man have not been investigated.

Phenoxybenzamine was used in these investigations as a tool for study of blockade of sympathetic impulses to the extremity. In doses of 2 to 4 mg, arterial administration of phenoxybenzamine completely blocked the constrictor response of forearm vessels to intra-arterial norepinephrine and epinephrine in eight subjects studied in this laboratory. The agent has long been known to block response of smooth muscle to sympathetic nerve stimulation and to circulating catecholamines. It does not block ganglia, or prevent contraction of smooth muscle in response to numerous other stimuli (1). Duff (23) showed that intra-arterial phenoxybenzamine increased blood flow in the hand after sympathectomy. He assumed that this indicated a direct vasodilating effect of the drug. An alternate explanation can be proposed —namely, that phenoxybenzamine blocked the constrictor response of the vessels to circulating catecholamines.

With regard to the vascular beds evaluated by forearm plethysmography, it has long been assumed that, since the forearm contains four times as much muscle as skin (24), recorded flow chiefly represents muscle blood flow. Cooper, Edholm and Mottram (25) have recently shown, using epinephrine iontophoresis, that muscle flow represents 80 per cent of total plethysmographically

recorded flow in the normal forearm, but that when total flow is increased by raising plethysmograph temperature, skin flow may represent 50 per cent of the total. In our experiments, therefore, muscle blood flow probably represents the majority of recorded flow in the intact forearm, but the relative proportions of muscle and skin flow in the forearm after phenoxybenzamine are unknown. It seems probable, however, that it is largely muscle flow which is increased in the "Dibenzylinized" forearm by induced acidosis or alkalosis. This probability is supported by the observations of Deal and Green (16) that blood flow through dog extremity muscle increases when either acid or alkaline solutions are infused directly into arteries supplying the muscle. Furthermore, uptake of radiosodium from deposits in the leg muscle of dogs pretreated with tetraethyl ammonium chloride increases with intravenous acid or alkaline infusions (26).

B. General circulatory effects. The mechanisms by which hypercapnia produces the demonstrated increase in cardiac output cannot be completely understood from the data presented. The vigorous respiratory movements accompanying elevation in blood CO_2 tension cannot be responsible, since, as shown above, comparable degrees of voluntary hyperventilation without change in arterial P_{CO_2} do not alter cardiac output. These data are not in keeping with the time-honored concept of the effectiveness of the respiratory movements of the thorax as a blood pump. Sechzer and co-workers (3) have recently demonstrated that inhalation of CO_2 in concentrations such as to raise end-expiratory CO_2 tension to 50 to 60 mm Hg was accompanied by increase in plasma concentrations of norepinephrine and epinephrine, so that these catecholamines may be responsible for the rise in heart rate, cardiac output, and blood pressure during hypercapnia.

The findings herein reported are at variance with the conclusions of Grollman (27) who studied the effect of hypercapnia on cardiac output in much the same manner 28 years ago and concluded that the changes observed after natural breathing of carbon dioxide mixtures are to be attributed to the mechanical aid which violent respiratory efforts exert on the circulation. Grollman's data included ten experiments on six individuals who breathed CO_2 and eight experiments on three individuals who voluntarily hyperventilated while breathing mixtures of CO_2 in air which maintained alveolar CO_2 concentration near control levels. In his subjects, hypercapnia produced only an 18 per cent increase in cardiac output, which was statistically insignificant. The different conclusion reached in the present study results from more extensive data, believed to be more significant.

The increased cardiac output and decreased total peripheral resistance accompanying hypercapnia indicate vasodilation, either active or passive, in some vascular beds (28). Elevation in blood CO_2 tension does not affect blood flow in the human calf or forearm (*vide supra*), areas representative of muscular circulation. Hypercapnia decreases flow through the hand (29, 30), where blood flow is largely cutaneous. In normal man, 13 per cent reduction in renal blood flow and 46 per cent increase in renal vascular resistance accompany inhalation of 10 per cent CO_2 (22). Splanchnic blood flow has not been measured in man with elevated CO_2 tension, but it increases during hypercapnia in the anesthetized, open-chest dog (31). Human brain blood flow nearly doubles during 7 per cent CO_2 inhalation, with increases averaging about 0.8 L of blood per brain per minute, a figure calculated from data of Schieve and Wilson (18), with assumed brain weight of 1,400 g. This rise in brain blood flow accounts for one-third of the increase in cardiac output observed during hypercapnia. Presumably increased splanchnic blood flow accounts for most of the remainder.

It would appear that elevation in blood CO_2 tension, rather than fall in pH, is the important factor in the rise in cardiac output accompanying hypercapnia, since it has been shown above that decrease in pH of similar magnitude, without change in arterial P_{CO_2}, during infusion of ammonium chloride or lactic acid, had no effect on cardiac output.

The effects of hypocapnia on cardiac output and arterial pressure have been summarized by Burnum and co-workers (4). They demonstrated that 50 per cent reduction in arterial P_{CO_2}, induced in normal subjects by vigorous voluntary overbreathing for 1 minute, was accompanied by rise in cardiac output from a control average of 7.5 to 10.5 L per minute, by blood pressure re-

TABLE IX

Circulatory effects of changes in arterial CO_2 tension

	7% CO_2 Increased arterial P_{CO_2}	Hyperventilation Decreased arterial P_{CO_2}*	Hyperventilation Unchanged arterial P_{CO_2}
	%	%	%
Cardiac output	+45†	+50†	−0.6
Heart rate	+24†	+63†	+4
Stroke volume	+20†	−13	+3
Blood pressure	+18†	−26†	+5
Systemic vascular resistance	−23†	−45†	+6

* Data from Burnum, Hickam and McIntosh (4).
† Statistically significant change ($p < 0.01$).

duction which averaged 23 mm Hg, and by 45 per cent decrease in systemic vascular resistance.

General circulatory responses to change in arterial carbon dioxide tension and to vigorous respiratory movements without change in arterial P_{CO_2} are summarized in Table IX.

SUMMARY

In human subjects, alkalemia produced by intravenous infusion of hypertonic sodium bicarbonate was accompanied by significant increase in cardiac output and decrease in total peripheral resistance. Acidemia produced by intravenous ammonium chloride or lactic acid solutions was not associated with significant change in cardiac output.

Neither induced alkalosis nor acidosis caused change in blood flow through the intact human forearm. With sympathetic vasoconstrictor impulses to the forearm blocked by phenoxybenzamine (Dibenzyline), both acidosis and alkalosis produced increases in forearm blood flow, the larger increase being associated with alkalosis.

In the intact extremity, blood flow did not change during inhalation of 5 to 7 per cent CO_2 for 5 to 10 minutes. Following blockade of sympathetic impulses by intra-arterial injection of phenoxybenzamine, CO_2 inhalation produced a significant increase in blood flow and decrease in vascular resistance. Hypocapnia, induced by voluntary hyperventilation, was accompanied by 20 per cent increase in blood flow in the intact forearm.

Inhalation of 7 per cent carbon dioxide in air for 7 minutes, in 16 experiments on 10 normal human subjects, produced a 45 per cent increase in cardiac output accompanied by a rise in blood pressure and heart rate. Comparable degrees of voluntary hyperventilation without change in arterial CO_2 tension caused no changes in these functions.

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THE ROLE OF CHELATION AND OF HUMAN PLASMA IN THE UPTAKE OF SEROTONIN BY HUMAN PLATELETS *

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Recent studies in this laboratory of platelets from patients with rheumatoid arthritis and nonrheumatoid inflammatory states (1) have emphasized the importance of the transport system or systems concerned with absorption and release of serotonin by platelets. There is no agreement or definite knowledge concerning such transport mechanisms at present. Born, Ingram and Stacey (2) reported a positive correlation between serotonin (5HT) absorption and ATP content in platelets, and Baker, Blaschko and Born (3) observed that 5HT and ATP were present in the same cytoplasmic fraction of platelets. Hughes, Shore and Brodie (4) felt that the rapid inhibition of uptake of serotonin produced by reserpine suggested blocking of an energetic transport mechanism for absorption, whereas the slow rate of release of serotonin from such platelets was consistent with passive diffusion. As regards an active absorption mechanism, Sano, Kakimoto and Taniguchi (5) noted a stimulatory effect of glucose on absorption of serotonin by rabbit platelets; they attributed this to ATP formed during glucose oxidation and suggested that ATP supplied the energy for absorption. However, inhibition of glucose metabolism did not result in slow release of serotonin as might have been expected with passive diffusion. An additional enzymatic mechanism for release was postulated therefore. Weissbach, Bogdanski and Udenfriend (6) and Weissbach, Redfield and Titus (7) called attention to the role of phosphates and potassium in the uptake of serotonin by saline suspensions of human platelets and to the inhibitory effect of cardiac glycosides on this system. They noted evidences for different mechanisms of uptake in different systems *in vitro*.

In the present study the problem of uptake of 5HT was explored by two types of experiments. I. Dye reduction by human platelets [1]; this method was used chiefly as a rapid screening test of *in vitro* systems in exploring for those consistently altered by addition of 5HT. Such a system, defined by this method, was explored further by experiments of type II, the results of which could be subjected to more specific interpretation than could the redox studies. II. Measurable uptake of 5HT by human platelets *in vitro*.

Enzyme inhibitors which altered redox activity of platelets did not necessarily produce parallel alteration of 5HT effect on redox activity, indicating as might be expected that 5HT effect on redox activity was only one event added to the many metabolic reactions contributing to overall redox activity of the intact platelet. However and more specifically, platelet systems in which redox activity was altered by addition of 5HT showed parallel alteration in ability to take up added 5HT *in vitro*. Evidence obtained by the combined approach suggested that bound divalent cations of a nondialyzable plasma factor or factors were concerned with serotonin uptake in this system.

METHODS

Platelets were obtained from hospitalized patients with no evidence of primary hematologic, metabolic or malignant disease, and in most instances with no evidence of either rheumatoid or nonrheumatoid inflammatory states. Blood was drawn as previously noted (10), using disodium EDTA as anticoagulant.[2] Platelets were

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[1] Using 2,6-dichloroindophenol (DIPH). Intact platelets were used in all studies because in platelets, unlike most tissues, dehydrogenase activity appears to be dependent upon an intact cell structure (8). This finding of Koppel and Olwin was confirmed here and was not altered by separation of stroma or by inhibition of DPNase by nicotinamide, in experiments patterned after those of Alivisatos and Denstedt (9).

[2] In a few experiments, native platelets were harvested from ambulatory subjects without use of anticoagulants. Dye reduction by these platelets was of the same general order of magnitude as that of platelets harvested from anticoagulated blood.

harvested by differential centrifuging as in earlier studies (10). Initial suspension of the platelet buttons was in physiologic saline to a stock suspension having a standard photometric density of 0.500 [optical density (O.D.) 420 mμ on a Coleman Junior spectrophotometer, saline blank]. Density readings were demonstrated to bear a linear relationship to serial dilutions of platelet suspensions.

I. Dye reduction by platelets. The method used was patterned closely after that of Chaudhuri and Martin (11) who worked with leukocytes. Identical readings were obtained with and without mineral oil seal, so mineral oil was omitted. To a 13 × 100 mm glass tube, aligned in a rack behind its corresponding 12 × 75 mm cuvet, was added 1.0 ml of DIPH (stock 2.5 mg DIPH per 100 ml 0.85 per cent NaCl). To each cuvet was added 1.0 ml of platelet suspension (now diluted as desired to O.D. $_{420\ m\mu} = 0.125$) and 0.5 ml of either 5HT [3] or its solvent or a chelator in the same solvent. Optical densities were read serially on a Coleman Junior spectrophotometer at 600 mμ, mixing by pouring rapidly back and forth the contents of glass tube and its corresponding cuvet immediately before a zero reading was recorded and placing the cuvet immediately in a 37° C water bath. Readings were then recorded at 10, 20, 30 and 55 minutes after the zero reading. All reaction mixtures were run in duplicate (with close checks obtained uniformly and easily) and read against a blank of the same mixture with buffer substituted for DIPH.

Test substances—e.g., enzyme inhibitors—were incorporated in the platelet suspension or the DIPH solution as part of the diluent used for final dilution of these reactants. Platelets were kept iced and in siliconed glassware from time of first centrifugation until addition to cuvets. Basic platelet diluent was 0.85 per cent NaCl.

[3] In creatinine sulfate form, as generously furnished by Abbott Laboratories and by the Upjohn Co.

Basic DIPH diluent was a buffered balanced salt solution [4] at pH 7.4, except as noted in a few experiments in which phosphate was eliminated from the system; here either veronal-HCl or Tris-HCl buffer (initial 6.7×10^{-3}M) was used, each with added NaCl to 0.75 per cent and KCl to 0.075 per cent.

II. Measurable uptake of serotonin by platelets. Conditions under which addition of 5HT resulted in enhancement of DIPH dye reduction by platelets were adapted with appropriate controls to a system patterned after that used in earlier 5HT recovery experiments (1). Final flask volumes totaled 5 ml. All tests were run in duplicate or triplicate in a Warburg shaker water bath at 37° C. 5HT and/or a chelator was added after one minute of equilibration at 37° C, and each flask was placed in ice after exactly 20 minutes of further incubation. Separation and washing of platelets was carried out at 5° C as in earlier studies (10). Supernatant fluid obtained after centrifugation of the incubation medium was measured for per cent dye reduction in a Coleman Junior spectrophotometer at 600 mμ, using as blank the supernatant fluid of a platelet flask from which DIPH had been omitted; standard baseline was the O.D. $_{600\ m\mu}$ of identically-diluted DIPH without platelets. Platelets were then extracted and 5HT content measured fluorometrically as in previous studies (10). The presence of DIPH either within or bound to the washed platelets was evidenced by appearance of blue-dyed proteinaceous debris at the butanol-aqueous interface on first extraction of platelets with alkaline butanol. The intensity of blue dye in the debris appeared to be proportional to the DIPH remaining oxidized in the supernate which was

[4] Ten ml stock solution (7.5 per cent NaCl, 0.75 per cent KCl, 0.1 per cent Na_2HPO_4, 0.12 per cent KH_2PO_4, 0.05 per cent K_2HPO_4) and 7 ml 1 per cent Na_2HPO_4 per 100 ml, aqueous (total phosphate concentration, 6.7×10^{-3}M).

TABLE I

Effect of various test substances on 2,6-dichloroindophenol (DIPH) dye reduction by human platelets in presence of plasma

Added to basic mixture*	In final conc. (M)	% Dye reduced, test − % dye reduced, control			
		10†	20	30	55
Cyanide	2×10^{-4}	+5.7	+9.4	+11.8	+16.3
Cyanide	1×10^{-2}	−11.1	−20.9	−27.7	−23.9
Arsenate‡	1×10^{-2}	−12.9	−18.5	−19.1	−17.2
Fluoride‡	6×10^{-3}	− 7.9	− 8.4	− 6.3	− 3.6
Malonate‡	3.4×10^{-3}	+ 8.7	+13.6	+12.8	+ 9.5
2,4-Dinitrophenol‡	9×10^{-4}	− 7.3	− 5.6	+ 1.9	+13.0
Iodoacetate‡	3.2×10^{-3}	−35.3	−52.4	−59.4	−61.0
Disodium EDTA‡	1.2×10^{-2}	+17.1	+21.9	+19.5	+13.8
Disodium calcium EDTA‡	1.2×10^{-2}	+ 1.2	+ 1.7	+ 1.2	+ 0.7
5HT creatinine SO_4‡	5×10^{-4}	+13.2	+16.3	+13.8	+ 8.4

* Basic reaction mixture (final concentrations): 1.0 ml platelet suspension (O.D. $_{420\ m\mu} = 0.125$) in physiologic saline plus glucose (1×10^{-3}M); 4 per cent added (in platelet suspension) platelet-free EDTA plasma (disodium EDTA final 1.5×10^{-4}M); 0.5 ml balanced buffered salt solution (pH 7.4, phosphate 4×10^{-3}M); 1.0 ml DIPH dye (10 μg per ml balanced salt solution).

† Time in minutes after zero point of mixing.

‡ Sodium cyanide (2×10^{-4}M) present in both test and control.

removed prior to washing the platelets with saline and extracting.

RESULTS

I-A. Dye reduction by platelets. The effect of various added substances on dye reduction by platelets is summarized in Table I. Cyanide inhibition of dye reduction occurred at concentrations of 1×10^{-2}M but only in the presence of phosphate. Lower concentrations (2×10^{-4}M) as used in the basic reactant mixture for evaluation of 5HT redox effect (see below) accelerated activity mildly in the presence of glucose and phosphate.

In the presence of plasma, using a standard final reactant mixture of platelets (O.D. $_{420\ m\mu}$ = 0.050), glucose (1×10^{-3}M), cyanide (2×10^{-4}M) in buffered balanced salt solution (phosphate 4×10^{-3}M), and 4 per cent added platelet-free EDTA plasma (disodium EDTA 1.5×10^{-4}M), 5HT consistently and significantly enhanced dye reduction (Figure 1), unlike several related substances (Table II). This will be referred to hereinafter as the "5HT redox effect." The 5HT effect was dependent upon the presence of platelets; the same system without platelets had minimal redox activity which was not altered by addition of 5HT. Alteration of the 5HT redox effect by added substances is summarized in Table III. Norepinephrine could not be substituted for 5HT with the same effect. Apparent partial inhibition of 5HT redox effect by large concentrations of reserpine appeared to result from an increase in dye reduction by platelets in presence of reserpine without a corresponding further increase on adding

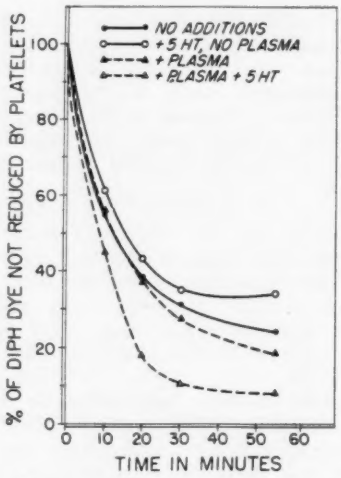

FIG. 1. EFFECT OF PLASMA AND 5HT ON DIPH DYE REDUCTION BY HUMAN PLATELETS. Basic reactant mixture (final concentrations): 1.0 ml platelet suspension in glucose 1×10^{-3}M in saline, O.D. $_{420\ m\mu} = 0.050$, with (or as indicated without) 4 per cent added platelet-free EDTA plasma (disodium EDTA 1.5×10^{-4}M); 0.5 ml salt solution or 5HT (serotonin creatinine sulfate, 5×10^{-4} M) in balanced buffered salt solution (pH 7.4, phosphate 4×10^{-3}M); 1.0 ml DIPH dye 10 μg per ml NaCN (2×10^{-4}M) in balanced salt solution.

5HT. This applied also to apparent inhibition of 5HT redox effect by potential chelators including malonate and disodium EDTA [5] (Figure 2, A and B), in contrast to the absolute inhibition produced

[5] Disodium EDTA as (Ethylenedinitrilo) tetraacetic acid disodium salt, Eastman, or as generously supplied as Endrate Disodium and Endrate Disodium Calcium by Abbott Laboratories.

TABLE II

Comparison of effect of serotonin (5HT) and related substances on DIPH dye reduction by human platelets in presence and absence of plasma

Added to basic mixture*	In final conc. (M)	% Dye reduced, test − % dye reduced, control			
		10†	20	30	55
A. 5HT creatinine SO_4	5×10^{-4}	−11.5	−11.0	− 8.3	− 4.6
5HIAA	5×10^{-4}	+ 0.3	− 0.8	− 1.6	− 3.0
Creatinine	5×10^{-4}	+ 1.3	+ 1.6	+ 1.5	+ 1.4
Tryptamine HCl	5×10^{-4}	− 9.2	−12.6	−14.8	−17.2
B. 5HT creatinine SO_4	5×10^{-4}	+16.0	+14.3	+ 8.8	+ 4.9
5HIAA	5×10^{-4}	+ 1.8	+ 1.4	+ 0.3	− 0.3
Creatinine	5×10^{-4}	− 0.6	+ 0.3	+ 0.2	+ 0.1
Tryptamine HCl	5×10^{-4}	+ 4.7	+ 4.2	+ 1.7	+ 1.0

* Basic mixture same as in Table I except plasma omitted in A; 4% plasma added in B, as in Table I. Sodium cyanide (2×10^{-4}M) present in both test and control.

† Time in minutes after zero point of mixing.

TABLE III

Effect of various test substances on 5HT redox effect in human platelets*

Added to basic mixture†	In final conc. (M)	5HT redox effect, test − 5HT redox effect, control			
		10‡	20	30	55
Cyanide	1×10^{-2}	+ 0.2	+ 2.2	+ 4.3	+ 7.1
Arsenate	1×10^{-2}	− 0.3	+ 0.1	+ 2.6	+ 2.1
Fluoride	6×10^{-3}	− 9.4	−17.9	−19.7	−21.7
Malonate	3.4×10^{-3}	−16.1	−16.1	−11.1	− 7.7
2,4-Dinitrophenol	9×10^{-4}	−16.6	−20.5	−18.3	−10.7
Iodoacetate	3.2×10^{-3}	− 8.9	−11.5	− 7.0	− 4.4
Disodium EDTA	1.2×10^{-2}	−17.1	−21.9	−19.5	−13.8
Disodium calcium EDTA	1.2×10^{-2}	+ 0.3	− 1.5	− 1.1	− 0.6
Reserpine	40 μg/ml	− 9.0	− 9.8	− 8.1	− 4.5
Propylene glycol	2.4 mg/ml	+ 1.9	+ 1.8	+ 0.2	+ 0.2

* Defined in text as the enhancement of DIPH dye reduction by platelets when serotonin (5HT) was added to the basic reactant mixture. Calculated here as per cent DIPH dye reduced in presence of 5HT minus per cent dye reduced in basic mixture alone.

† Basic reactant mixture as for Table I, including cyanide 2×10^{-4}M.

‡ Time in minutes after zero point of mixing.

by fluoride and by 2,4-dinitrophenol (Figure 2, C and D). In further contrast, iodoacetate which markedly inhibited dye reduction by platelets (Table I) only partially inhibited 5HT redox effect (Table III); arsenate and cyanide (1×10^{-2}M) which inhibited dye reduction did not alter 5HT redox effect. Calcium EDTA[5] did not alter dye reduction by platelets or the 5HT redox effect, in contrast to disodium EDTA.

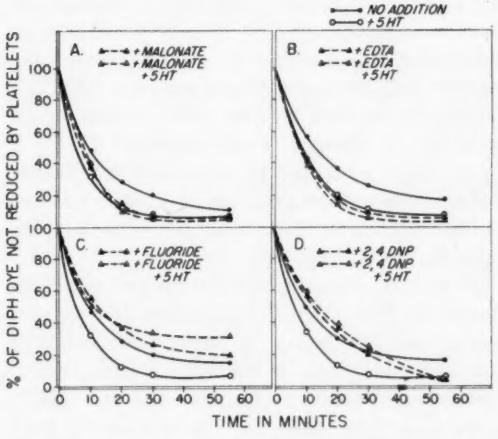

FIG. 2. ALTERATION OF 5HT REDOX EFFECT BY (A) MALONATE, (B) DISODIUM EDTA, (C) FLUORIDE, AND (D) 2,4-DINITROPHENOL. Results, I-A. Basic reactant mixture, same as Figure 1, including plasma. Additions as indicated above, incorporated in DIPH dye (A, C and D) or in platelet suspension (B) to final concentration: (A) malonic acid, neutralized with NaOH, 3.4×10^{-3}M; (B) disodium EDTA, 8.6×10^{-4}M; (C) sodium fluoride, 6×10^{-3}M; (D) 2,4-dinitrophenol, 9×10^{-4}M.

There was a linear relationship between concentration of either added 5HT or (within range 4 to 8×10^{-4}M) disodium EDTA and enhancement of dye reduction by platelets (Figure 3). The effect of added 5HT (5×10^{-4}M) was roughly equivalent to that of disodium EDTA (6.6×10^{-4}M) in this regard.[6] In studying the effect of

[6] It will be noted in Figure 3 that at lower concentrations of disodium EDTA, including the 1.5×10^{-4}M final concentration resulting from addition of EDTA plasma to some reactant mixtures, the linear relationship was no longer maintained. The effect of this concentration on dye reduction or on 5HT redox effect was relatively slight.

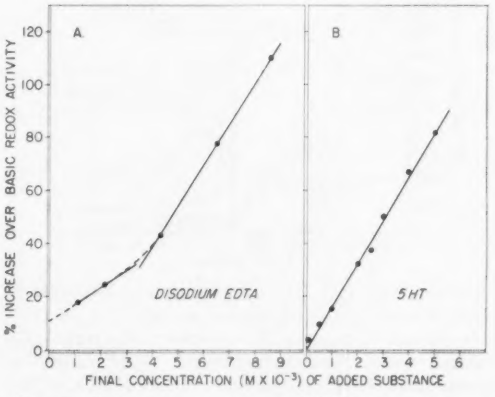

FIG. 3. PER CENT INCREASE IN DIPH DYE REDUCTION BY HUMAN PLATELETS AT 20 MINUTES WITH VARYING AMOUNTS OF ADDED (A) DISODIUM EDTA OR (B) 5HT. Basic reactant mixture, same as in Figure 1, including plasma. Disodium EDTA and 5HT (serotonin creatinine sulfate) in final concentrations as indicated.

added Ca^{++} on the 5HT redox effect, increasing turbidity and precipitate during incubation of some systems suggested possible formation of calcium soaps. A system patterned in principle after that of Gomori (12) was designed to investigate this possibility by omitting DIPH from the system and recording turbidities at 420 mμ. While formation of calcium soaps was not ruled out in view of the secondary and considerable rise in turbidity noted in Figure 4A, it was apparent from experiments omitting platelets that formation of calcium phosphate was a more likely explanation of events. Calcium phosphate was solubilized by 5HT (Figure 4B).

1-B. The plasma factor necessary for 5HT redox effect. Enhancement of dye reduction by either 5HT or disodium EDTA occurred only in the presence of added plasma. The plasma effect was maximal at plasma concentrations of 2 to 4 per cent (Figure 5). Plasma dialyzed exhaustively against a balanced buffer, pH 7.4 (phosphate or veronal or Tris), was fully active in the system. Autologous plasma was as active as homologous plasma. Refrigerated aging of plasma for at least 1 week did not decrease its activity in the system. Rapid freezing and thawing six times had no effect on activity of plasma in the system,

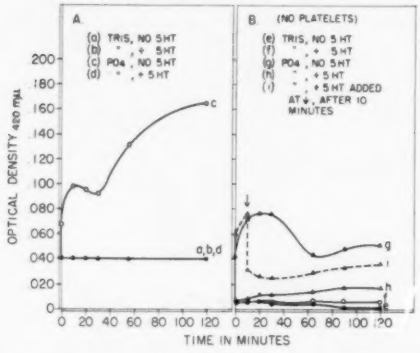
A.
(a) TRIS, NO 5HT
(b) " , + 5HT
(c) PO4 , NO 5HT
(d) " , + 5HT
B. (NO PLATELETS)
(e) TRIS, NO 5 HT
(f) " , + 5HT
(g) PO4 , NO 5HT
(h) " , + 5HT
(i) " , + 5HT ADDED AT ↓, AFTER 10 MINUTES
OPTICAL DENSITY 420 mμ
TIME IN MINUTES

FIG. 4. EFFECT OF 5HT ON TURBIDITY OF MIXTURES OF CALCIUM ACETATE AND PHOSPHATE BUFFER, (A), IN PRESENCE OF PLATELETS, AND (B) WITHOUT PLATELETS. No plasma present. Final concentrations: 1.0 ml platelets or glucose-saline + Ca^{++}; 1.0 ml buffer + NaCN (2×10^{-4}M) (Tris or phosphate buffer 4×10^{-2}M + NaCl 0.03% + KCl 0.003%); 0.5 ml buffer or 5HT, serotonin creatinine sulfate) 5×10^{-4}M in buffer indicated above. Platelets suspended in glucose initial 2.5×10^{-2}M in saline + calcium acetate initial 12.5×10^{-3}M and held in ice bath for several hours before use.

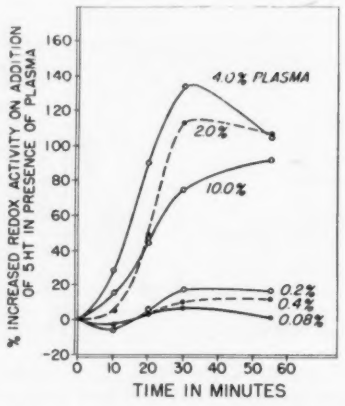

FIG. 5. COMPARISON OF DEGREE OF ENHANCEMENT OF DYE REDUCTION BY HUMAN PLATELETS BY 5HT IN PRESENCE OF VARYING CONCENTRATIONS OF PLATELET-FREE EDTA PLASMA (EDTA 3.8×10^{-3}M IN ORIGINAL PLASMA). Basic reactant mixture as in Figure 1. 5HT (as creatinine sulfate salt) added in final 5×10^{-4}M concentration.

nor did heating to 37° or to 56° C for 30 minutes diminish its activity. Activity in the system was decreased after 15 minutes at 80° C and destroyed by 5 minutes at 100° C. Butanol extraction of plasma at 25° C was without effect on activity of the supernatant plasma. Euglobulin obtained by isoelectric precipitation at pH 5.8 could not be substituted for plasma, nor could the remaining supernatant plasma (after restoration to pH 7.4), raising the question of irreversible damage to the factor by pH change. Cohn fractions[7] II, IV-1, IV-4, and V could not be substituted for plasma. Trial of other Cohn fractions of plasma was unsatisfactory due to their denatured state as indicated by partial insolubility in saline.

Phosphate in final concentration and at pH indicated by Weissbach and colleagues (6) for promotion of 5HT uptake by platelets could not be substituted for plasma in the redox system. Elimination of phosphates by dialysis of plasma against veronal or Tris buffer did not abolish the 5HT redox effect.

II. Measurable uptake of serotonin by platelets. Results are summarized in Tables IV and V, including single readings on per cent reduction of

[7] Obtained from E. R. Squibb & Sons, through the courtesy of Dr. J. N. Ashworth of the American National Red Cross.

TABLE IV

Influence of plasma, chelators and magnesium on the uptake of added serotonin by human platelets in vitro

Flask mixture*	5HT†	DIPH redox dye‡
to 5 ml vol	*Total μg*	*% reduction*
a. PT (0.104) +G +B +CN +5HT (5×10^{-4}M) +D	2.8 (d 8.1%)	58.1
Same + 4% PL	4.1 (d 5.6%)	91.3
PT (0.104) +G +B +CN +5HT (5×10^{-5}M) +D	1.5 (d 11.5%)	61.7
Same + 4% PL	2.5 (d 5.8%)	92.7
b. PT (0.102) +G +B +CN +5HT +D	1.4 (t 7.9%)	30.0
Same + 4% PL	1.9 (t 4.4%)	61.2
Same + 4% PL +E	2.3 (t 4.7%)	74.7
c. PT (0.104) +G +B +CN +5HT +D +4% PL +E	2.0 (t 4.7%)	77.8
Same +Mg	1.4 (t 4.3%)	57.6
Same +Mg except no E	2.0 (t 9.3%)	64.8
d. PT (0.104) +G +B +CN +5HT +D +4% PL	1.6 (t 0%)	54.4
Same +Mg	1.6 (t 4.5%)	54.0
Same (no Mg) +CE	1.6 (t 5.8%)	51.8

* Final concentrations, except plasma EDTA: PT = platelet suspension (O.D. 420 mμ) in physiologic saline. G = glucose, 1×10^{-3}M. B = balanced buffered salt solution ($PO_4 = 4 \times 10^{-3}$M; pH 7.4). CN = sodium cyanide, 2×10^{-4}M. 5HT = serotonin creatinine sulfate, 5×10^{-5}M unless otherwise stated. D = 2,6-dichloroindophenol (DIPH), 20 μg per ml. PL = platelet-free plasma with disodium EDTA 3.8×10^{-3}M as harvested. E = disodium EDTA, 1.2×10^{-2}M. CE = disodium calcium EDTA, 1.2×10^{-2}M. Mg = $MgSO_4 \cdot 7H_2O$, 8.1×10^{-3}M.

† Since each experiment was a comparative unit, endogenous and added 5HT are expressed as a combined total, as 5HT creatinine sulfate, with per cent deviation of duplicate (d) or triplicate (t) flasks in parentheses.

‡ 100 minus $\frac{\text{O.D. 600 m}\mu\text{ of supernatant fluid}}{\text{standard original O.D. 600 m}\mu \times 100} \times 100$ = % reduction DIPH dye.

DIPH dye at 20 minutes in the supernatant fluid of reaction flasks where this was feasible (Table IV). The 5HT uptake was observed to correlate with degree of reduction of dye. Enhanced uptake of 5HT occurred under conditions comparable with those in which 5HT redox effect was observed (Table IV, a), and this was further enhanced by addition of disodium EDTA (b) but not by addition of disodium calcium EDTA (d). Addition of Mg^{++} was without effect in the system (c, d) unless combined with chelators. Although Mg^{++} chelates have a lower stability constant than have Ca^{++} chelates, the small amount of Ca^{++} displaced in arriving at an equilibrium after addition of Mg^{++} to disodium calcium EDTA sufficed to produce the gel which was seen regularly on in-

TABLE V

Influence of phosphates on uptake of serotonin by human platelets, with and without added plasma, DIPH redox dye and chelators, in vitro

Flask mixture (to volume 5 ml)*	Total μg 5HT†
a. PT (0.188) +B +5HT (5×10^{-6}M)	4.5 (d 2.3%)
Same + (W)B; no PL	7.7 (d 8.0%)
b. PT (0.188) +G +B +5HT (5×10^{-6}M)	2.5 (d 1.8%)
Same + (W)B; no PL	3.5 (d 1.3%)
c. PT (0.104) +G +B +CN +5HT +D +4% PL	2.2 (t 4.5%)
Same + (W)B	2.0 (t 8.5%)
Same + (W)B except no PL	1.1 (t 11.2%)
d. PT (0.105) +G +B +CN +5HT +D +4% PL	2.0 (t 2.3%)
Same + (W)B	1.8 (t 5.0%)
Same + (W)B +E	1.8 (t 2.6%)

* Final concentrations, except plasma EDTA: PT = platelet suspension ($O.D._{420\ m\mu}$) in physiologic saline. B = balanced buffered salt solution (pH 7.4, phosphate 4×10^{-3}M). (W)B = phosphate buffer 5×10^{-2}M, as used by Weissbach and co-workers (6). 5HT = serotonin creatinine sulfate (5×10^{-5}M unless otherwise stated). PL = platelet-free plasma with disodium EDTA 3.8×10^{-3}M as harvested. G = glucose (1×10^{-3}M). CN = sodium cyanide (2×10^{-4}M). D = 2,6-dichloroindophenol (DIPH), 20 μg per ml. E = disodium EDTA, 1.2×10^{-2}M.

† Same as for Table IV.

troduction of excess free Ca^{++} into the *in vitro* system used in these experiments. The gel prevented measurement of uptake of 5HT. However, when Mg^{++} was added with disodium EDTA, uptake of 5HT was sharply curtailed and redox activity was reduced (Table IV, c).

Addition of phosphate buffer in concentration 5×10^{-2}M and at pH 7.0, as used by Weissbach and colleagues (6), to human platelets in balanced salt solution (phosphate 6.7×10^{-3}M) resulted in markedly enhanced uptake of 5HT (Table V, a) as noted by these investigators. The degree of enhancement was reduced by addition of glucose to the system (Table V, b). On completion of the system by addition of plasma, cyanide and DIPH, no enhancement of uptake occurred on addition of the higher concentration of phosphate, and reduction rather than enhancement of 5HT uptake by platelets occurred when the higher concentration of phosphate was substituted for added plasma (c). Disodium EDTA did not enhance 5HT uptake in presence of the higher concentration of phosphate (Table V, d), in contrast to the enhancement noted in presence of low concentrations of phosphate (Table IV, b).

DISCUSSION

The problem of serotonin uptake by human platelets was approached *1*) by a screening method which defined certain systems in which dye (DIPH) reduction by intact human platelets *in vitro* was consistently altered by adding 5HT in the presence of plasma ("5HT redox effect"), and *2*) by measuring uptake of 5HT by intact human platelets *in vitro* in the systems so defined.

The over-all data on effect of enzyme inhibitors on dye reduction by human platelets (Table I) reemphasized the active metabolic state of these cytoplasmic particles, as has been recognized previously and reviewed by Zucker and Borrelli (13). They were useful in the present studies chiefly as a basis of comparison (Tables I to III) for investigating the 5HT redox effect, as just defined. Agents which inhibited over-all ability of platelets to reduce DIPH dye did not necessarily inhibit to a comparable degree the enhancing effect of 5HT on dye reduction in the presence of plasma (see Results, I-A). This emphasized well that over-all redox activity is a cumulative result of many metabolic activities and that alteration of redox activity by 5HT in the presence of plasma was but one added factor in the over-all picture. The observation might or might not have been meaningful in terms of reflecting change in the only known and previously measured physiologic activity of platelets in regard to 5HT—i.e., uptake of 5HT. However, the results of uptake studies showed a parallelism between 5HT redox effect and 5HT uptake in that both were accelerated by added disodium EDTA, both were unaffected by disodium calcium EDTA, and both were sharply retarded by magnesium in the presence of disodium EDTA (but not by magnesium alone). In neither type of experiment were these effects observed in absence of plasma, and substitution of nonphysiologically high concentrations of phosphate (5×10^{-2}M) for plasma resulted in decreased rather than enhanced uptake of 5HT. This emphasized the comments of Weissbach and co-workers (*7*) regarding the existence of different mechanisms for uptake in different systems *in vitro*. The high concentrations of phosphate found by these investigators (and confirmed here and elsewhere) to enhance uptake of 5HT in saline suspension of human platelets *in vitro* showed diminishing ability to enhance uptake when glucose was added to the system and retardation of uptake when cyanide and a hydrogen acceptor were further added. However, when plasma was present in the system, addition of the high concentration of phosphate was without any effect on uptake. Since the plasma factor or factors were active after prolonged dialysis, either large molecules per se or small substances bound to large molecules seemed implicated in uptake under the particular *in vitro* conditions employed. Two findings were then pertinent in this regard: *1*) 5HT could solubilize calcium phosphate; *2*) in presence of plasma, both the 5HT redox effect and 5HT uptake by human platelets in presence of plasma were sharply curtailed when another divalent cation (Mg^{++}) was first added and then chelated with disodium EDTA. This suggested the possibility that in this *in vitro* system 5HT complexed with a divalent ion bound to a large molecular plasma component as part of the process of uptake by platelets. This could be prevented by prior complexing of 5HT with free divalent ions and then binding with disodium EDTA. Whether these observations have any bearing on

the earlier hypotheses of Woolley (14, 15) regarding a tissue "portering substance," which could combine with serotonin and with calcium ions and diffuse across cellular membranes, must await further studies. The nature of the plasma factor or factors influencing serotonin uptake in the present studies is being investigated currently, as well as the presence of the factor or factors in various human disease states related to connective tissue.

SUMMARY

1. The problem of uptake of serotonin by human platelets was investigated by *1*) a screening method which measured dye (2,6-dichloroindophenol) reduction by intact platelets, and *2*) measurable uptake of serotonin by intact platelets, all in the presence of human plasma. The effect of various agents on these *in vitro* systems was observed.

2. Enhancement of dye reduction by platelets in the presence of serotonin and a plasma factor or factors ("5HT redox effect") correlated with increased uptake of serotonin by intact platelets.

3. Serotonin was shown to solubilize calcium phosphate *in vitro*. Prior mixing of serotonin with Mg^{++} followed by chelation with disodium EDTA markedly diminished both 5HT redox effect and uptake of 5HT by intact human platelets in the presence of plasma. Disodium calcium EDTA was without effect, as was Mg^{++} alone.

4. The possibility is entertained that in the presence of a factor present in human plasma, serotonin is taken up by human platelets as a complex with a divalent cation bound to a heat-stable, nondialyzable plasma factor.

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STUDIES ON DIGITALIS. IV. OBSERVATIONS IN MAN ON THE EFFECTS OF DIGITALIS PREPARATIONS ON THE CONTRACTILITY OF THE NON-FAILING HEART AND ON TOTAL VASCULAR RESISTANCE

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The observations that digitalis exerts a positive inotropic effect on the cat papillary muscle (1) and on the tortoise ventricle (2) led to the view, now generally accepted, that the salutary clinical and hemodynamic effects of this drug in congestive heart failure result primarily from its direct stimulation of myocardial contraction (3). This concept has received support from hemodynamic studies in patients with heart failure, demonstrating that digitalization results in an augmentation of the cardiac output and a lowering of the end-diastolic pressure of the failing ventricle (4–9). Considerable confusion still exists, however, concerning the effects of digitalis on the non-failing human heart, since in the absence of clinical heart failure acute digitalization either depresses the cardiac output slightly or produces no significant change in any hemodynamic parameter (6, 8, 10–14). Such observations have led to the contention that digitalis does not stimulate the non-failing human heart (12, 13, 15, 16).

In the present investigation the direct effects of digitalis preparations on the contractile force of the non-failing human heart were measured with the Walton-Brodie strain gage arch (17). This instrument has been extensively utilized in the study of the effects of drugs on the heart both in the dog (18) and in man (19, 20) and has also been found useful for monitoring myocardial contractile force during cardiac operations (21). In addition, observations were made to ascertain whether or not digitalis preparations have any direct action on the human systemic vascular bed.

METHODS

Digitalization was carried out during cardiovascular operations in 21 patients (Table I). In 18 of these the drug was administered in the course of an open operation during total cardiopulmonary bypass. These 18 patients ranged from 4 to 40 years and averaged 22 years of age. Sixteen of the patients had atrial septal defects and 2 had valvular pulmonic stenosis. None of the patients had a history of congestive heart failure, and although several patients had experienced mild limitation of exercise, in none was this limitation even moderately severe. All were studied by cardiac catheterization before operation, and moderate pulmonary hypertension (systolic pressure greater than 40 mm Hg) was present in 3 of the 16 patients with atrial septal defect. In several of the subjects with this anomaly a small systolic pressure gradient between the right ventricle and the pulmonary artery existed, presumably due to the excessive blood flow across the pulmonic orifice. However, in no patient was the right ventricular end-diastolic pressure elevated above the upper limit of normal (5 mm Hg). Fourteen of the 18 patients who were studied while they were on cardiopulmonary bypass received acetylstrophanthidin, the total dose ranging from 0.5 to 1.5 mg (average 1.15 mg). This dose equaled 0.013 to 0.036 mg per kg and averaged 0.026 mg per kg or 0.155 cat units per kg body weight. In general, smaller doses of acetylstrophanthidin were employed in the early portion of this study. When it became apparent that these doses resulted in no toxic effects they were gradually increased. Three of the other 4 patients digitalized while on cardiopulmonary bypass received 1.2 mg of lanatoside C, while the fourth patient received 0.80 mg of this glycoside. These doses equaled 0.021 to 0.026 mg per kg and averaged 0.022, or 0.088 cat units per kg body weight. It should be noted that the volume of blood circulating in these patients was increased by the volume of the extracorporeal circuit. However, it is not certain that the dilution of the glycosides by this increased circulating volume would modify the uptake of the glycosides by the myocardium.

Three adult patients with rheumatic heart disease received 1.0 to 1.5 mg acetylstrophanthidin immediately after mitral commissurotomy. None of these patients had experienced congestive heart failure and none had an elevated left ventricular end-diastolic pressure before operation, but two had moderate pulmonary hypertension.

For all patients, the pre-anesthetic medications included meperidine (25 to 75 mg), scopolamine (0.1 to 0.4 mg) and promethazine (15 to 50 mg). After intravenous thiopental induction, light general anesthesia was main-

TABLE I

Hemodynamic observations in patients digitalized during operation *

Patient	Age	Weight	Preoperative catheterization data			Dose	Cont. force incr.	Systemic vasc. resist. incr.
			RV press. s/d	PA press. s/d	P/S flow ratio			
	yrs	*kg*	*mm Hg*	*mm Hg*		*mg*	%	%
		Atrial septal defect				Acetylstrophanthidin		
C.R.	8	22	20/0	17/8	1.47	0.7	39	10
G.B.	13	32	25/4	22/7	1.38	1.0	43	13
D.G.	14	49	24/4	24/7	1.64	0.7	16	9
D.R.	18	51	46/5	35/10	1.54	1.0	62	35
E.R.	19	69	34/0	30/10	2.47	1.4	50	29
R.T.	24	65	23/3	20/8	3.03	1.5	117	58
M.T.	28	63	24/0	20/9	2.35	1.5	247	16
A.S.	30	69	32/0	28/10	1.74	1.5	57	29
N.R.	31	60	34/5	26/10	1.96	0.8	UN	10
C.T.	8	23	42/1	40/12	2.24	0.7	36	25
A.T.	27	53	40/2	40/8	2.35	1.5	UN	25
H.L.	28	43	72/2	64/28	1.81	1.4	327	36
		Atrial septal defect				Lanatoside C		
S.W.	11	31	32/0	28/10	2.41	0.8	10	10
J.B.	29	58	26/4	21/6	2.19	1.2	57	10
D.M.	34	58	30/0	26/10	2.04	1.2	UN	34
E.S.	40	57	32/5	30/11	3.91	1.2	26	0
		Pulmonic stenosis				Acetylstrophanthidin		
C.I.	4	17	110/5	11/5	1.0	0.5	30	0
D.A.	29	42	98/5	12/4	1.5	1.5	45	UN
		Mitral stenosis				Acetylstrophanthidin		
R.P.	19	67	48/5	48/22	1.0	1.0	16	
L.R.	30	60	30/4	27/16	1.0	1.25	30	
P.C.	45	69	65/7	65/42	1.0	1.5	25	

* RV press., right ventricular pressure; PA press., pulmonary artery pressure; P/S flow ratio, pulmonic systemic flow ratio; s/d, systolic/diastolic; Cont. force incr., increase in contractile force expressed as per cent above control levels; systemic vasc. resist. incr., increase in systemic vascular resistance expressed as per cent above control levels; UN, recording unsuitable for analysis.

tained with nitrous oxide-oxygen; a muscle relaxant, either succinylcholine or *d*-tubocurarine was also used. Thiopental and meperidine were administered intermittently in small doses.

In the 18 patients who were digitalized while on cardiopulmonary bypass, a median sternotomy was performed, the pericardium was opened and the strain gage arch was sutured to a convenient area on the right ventricle; the segment of myocardium between the two feet of the arch was stretched by about 50 per cent of its diastolic length. Total cardiopulmonary bypass was instituted with a rotating disc oxygenator and a single arm roller pump. Venous blood was drained from the venae cavae by gravity. A square-wave electromagnetic flowmeter (22, 23) in the arterial line was used to permit constant monitoring of the volume of arterial return and insured that the perfusion rate remained constant, ordinarily at 2.2 L per minute per m^2 BSA. The arterialized blood was returned into the cannulated femoral artery; the coronary flow was not interrupted. Arterial blood pressure was measured by means of a Statham pressure transducer through either a polyethylene catheter in the left radial artery or an indwelling needle in the brachial artery. Continuous recordings of the mean arterial and central venous pressures, myocardial contractile force, the electrocardiogram and the perfusion rate were made with a multichannel photographic oscillograph. Approximately 5 minutes after bypass was established and the right atrium or pulmonary artery had been opened, all of the hemodynamic parameters, as well as the myocardial contractile force, had stabilized and at this time the glycoside was administered by rapid injection into the arterial return line of the extracorporeal circuit.

In the 3 patients with rheumatic mitral stenosis who were studied, a left thoracotomy was performed and the strain gage arch sutured to the surface of the left ventricle. In one of these patients a second arch was attached to the surface of the right ventricle. Acetylstrophanthidin was administered intravenously during a five minute period after the commissurotomy had been performed and at a time when both the contractile force and arterial pressure were stable.

The contractile force recorded by the strain gage arch is dependent not only upon the force of contraction of the fibers to which the gage is attached but also the extent to which these fibers have been stretched and the

depth of the sutures which hold the gage to the myocardium (24). Since the electrical output of the arch and, therefore, the resultant deflection in the recording are directly proportional to the contractile force, the alterations in contractile force resulting from the administration of the digitalis preparations were expressed as relative changes from the control level rather than in absolute terms.

RESULTS

Contractile force. Recordings of contractile force were suitable for analysis in 12 of the 14 patients to whom acetylstrophanthidin was administered during cardiopulmonary bypass. The contractile force was increased in every patient, the values at the time of maximal effect ranging from 16 to 327 per cent and averaging 89 per cent greater than the contractile force prior to digitalization (Figures 1 and 2). Contractile force began to increase between 2 and 5 minutes after injection; the maximal change was noted 5 to 20 minutes (average 11.3) following injection. Termination of the intracardiac procedure and the cessation of perfusion prohibited determination of the duration of the inotropic effect. Contractile force recordings satisfactory for analysis were obtained in three of the four patients to whom lanatoside C was given. The maximal contractile force increases were 10, 57 and 26 per cent of the control values (average increase 31 per cent). The peak effect was observed 18, 20 and 27 minutes (average 22) after injection.

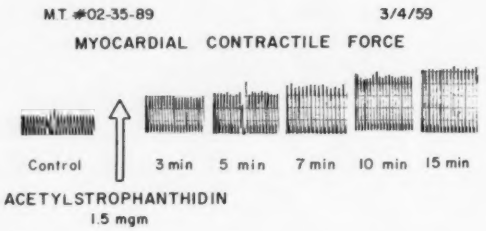

FIG. 1. MYOCARDIAL CONTRACTILE FORCE RECORDINGS IMMEDIATELY PRIOR TO AND AT INTERVALS FOLLOWING THE ADMINISTRATION OF 1.5 MG OF ACETYLSTROPHANTHIDIN TO A 28 YEAR OLD WOMAN WITH ATRIAL SEPTAL DEFECT. Augmentation of contractile force is evident 3 minutes after injection.

The maximal increases in left ventricular contractile force were 16, 30 and 25 per cent (average 24) in the three patients with rheumatic mitral stenosis to whom acetylstrophanthidin was given

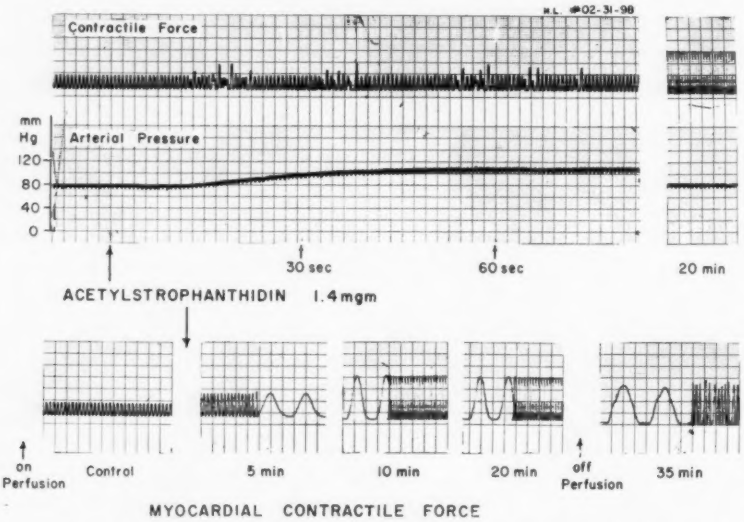

FIG. 2. CONTRACTILE FORCE AND ARTERIAL PRESSURE RECORDINGS IMMEDIATELY AND 20 MINUTES AFTER THE INJECTION OF 1.4 MG OF ACETYLSTROPHANTHIDIN, IN A 28 YEAR OLD WOMAN WITH AN ATRIAL SEPTAL DEFECT, ARE REPRODUCED IN THE UPPER TRACINGS. THE LOWER TRACINGS SHOW CONTRACTILE FORCE RECORDINGS BEFORE INJECTION AND AT INTERVALS AFTER ACETYLSTROPHANTHIDIN. THE FINAL RECORDING WAS OBTAINED AFTER BYPASS HAD BEEN COMPLETED, 35 MINUTES AFTER ADMINISTRATION OF THE DRUG.

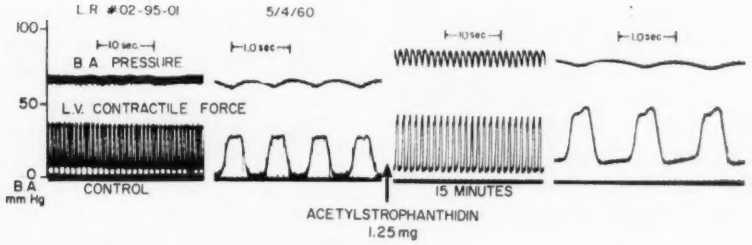

FIG. 3. MEAN BRACHIAL ARTERIAL PRESSURE (B.A.) AND LEFT VENTRICULAR (L.V.) CONTRACTILE FORCE RECORDINGS AT SLOW AND RAPID PAPER SPEEDS IN A 30 YEAR OLD MAN WITH RHEUMATIC MITRAL STENOSIS. The control recordings (left) were obtained just prior to the injection of 1.25 mg of acetylstrophanthidin and the recordings on the right were made 15 minutes after the completion of the injection. The scale on the left refers to the arterial pressure. The baseline of the contractile force recording was not maintained constant. Following acetylstrophanthidin, the elevation of arterial pressure and of contractile force and the slowing of the heart rate are evident.

(Figure 3). In the patient in this group (P.C.) in whom right ventricular contractile force was also recorded, a 33 per cent increase was observed.

No drugs were administered to 12 "control" patients with a variety of congenital cardiovascular anomalies in whom recordings of right ventricular contractile force were carried out during cardiopulmonary bypass. In one patient the contractile force increased by 8 per cent during the course of the perfusion; in five patients this parameter decreased by 5 to 15 per cent of the control value.

Systemic vascular resistance. Since the systemic perfusion rate was held constant during cardiopulmonary bypass, any change in arterial pressure reflects a change in systemic vascular resistance. Mean arterial pressure rose in all but one of the 13 patients who received acetylstrophanthidin while on cardiopulmonary bypass. The changes in systemic vascular resistance ranged from 0 to + 58 per cent and averaged + 23 per cent of the control level (Table I). The arterial pressure rose almost immediately—i.e., within 30 seconds of the injection (Figures 2 and 4). The pressure reached a peak in an average of 3 minutes following injection, and usually gradually declined toward control levels during the remainder of the perfusion.

Arterial pressure rose in three of the four patients who received lanatoside C and it remained unchanged in the fourth patient. The changes in systemic vascular resistance ranged from 0 to + 34 per cent and averaged + 14 per cent of control levels.

No systematic change in arterial pressure occurred during the course of the perfusion in any of the 12 patients to whom no drug was administered.

DISCUSSION

The Walton-Brodie strain gage arch records the isometric tension developed by the fibers to which it has been applied (24) and it is therefore

FIG. 4. RECORDING OF BRACHIAL ARTERIAL PRESSURE OBTAINED IN A 27 YEAR OLD WOMAN WHILE HER ATRIAL SEPTAL DEFECT WAS BEING REPAIRED DURING CARDIOPULMONARY BYPASS AT A CONSTANT SYSTEMIC PERFUSION RATE. Arterial pressure begins to rise 20 seconds after the injection. Seven minutes after injection the pressure has already fallen and 12 minutes after injection it is only slightly higher than the control level.

of value in the study of the direct effects of drugs and of other interventions on myocardial contractile force. In the present investigation, as well as in previous ones (19–21), it was found to be as applicable in man as in the experimental animal. Its presence did not interfere with the operation and no complication arose from its use; all of the patients whose data are recorded above survived operation.

Although alterations in the length of the fibers to which the gage is attached modify the contractile force which these fibers develop, such changes in length were minimized by initially stretching the segment of myocardium to which the gage was attached (24). Since the strain gage arch was placed on the right ventricle in most of the patients, other possible effects of changes in peripheral vascular resistance were minimized. In addition, most of the observations reported herein were carried out with the patients on cardiopulmonary bypass, at a constant systemic perfusion rate, and with the right side of the heart open to atmosphere and therefore completely decompressed. These conditions assured that the length of the segment of myocardium to which the gage was attached was constant.

A substantial body of evidence has now been accumulated which supports the contention that digitalis glycosides augment the contractile force of the non-failing dog heart. Wiggers and Stimson (25) and Cotten and Bay (24) found that these drugs shorten the duration of the period of isometric contraction. Walton, Leary and Jones (26), Cotten and Bay (24) and Cotten and Stopp (27) observed that digitalis augments the contractile force of the non-failing heart in experiments on dogs with open chests as well as in unanesthetized dogs with closed chests. Cotten and Stopp (27) showed that digitalis elevates the left ventricular function curve (28).

The experiments reported herein extend these findings to man and demonstrate that two digitalis preparations, acetylstrophanthidin, and lanatoside C, produce a substantial augmentation of the force of ventricular contraction when they are administered to patients who are not in heart failure. However, the patients with atrial septal defect and those with pulmonic stenosis undoubtedly had right ventricular dilatation and/or hypertrophy. In order to determine the actions of the digitalis preparations on myocardium which had not been subjected to an abnormal hemodynamic burden for a prolonged period of time, the effects of acetylstrophanthidin on left ventricular contractile force were recorded in three patients immediately following mitral valvulotomy; the drug elevated left ventricular contractile force in each instance. The relative increments in contractile force were somewhat smaller in these three studies than in the patients digitalized during cardiopulmonary bypass. The significance of this difference is difficult to interpret because: *1*) there was a wide scatter of the contractile force responses in both groups of patients; *2*) the conditions under which the drug was given differed appreciably in the two groups; and *3*) in the patients studied during cardiopulmonary bypass the strain gage arch was attached to the *right* ventricle, while in the patients with mitral stenosis, it was placed on the *left* ventricle.

Although digitalization was found to augment the force of contraction in chronically stressed non-failing hearts, the possibility that the stimulating effects are of different intensity in failing hearts or in entirely normal hearts has not been excluded. The concept that digitalis glycosides either have no effect on the non-failing heart or actually depress its contractility arose from the observations which showed that these drugs do not either modify or decrease the cardiac output, both in the dog (29, 30) and in man (6, 8, 10–14). This view received support from the experiments of Wedd and Blair who noted that, although digitalis did not stimulate contractions of strips of turtle ventricle, an augmentation of contraction did occur when digitalis was applied after the contractility of the muscle had been depressed by a variety of chemicals or by reduction of the pH (31). Furthermore, Olson recently reported that when the administration of digitoxin to dogs in experimental congestive heart failure resulted in clinical improvement, the abnormally elevated intrinsic viscosity of myosin which occurs in experimental heart failure was restored to normal; however, digitoxin did not alter the viscosity of the myosin of the non-failing heart (32). On the other hand, Kako and Bing found that digoxin and ionic calcium augmented the contractility of

actomyosin threads obtained from normal hearts as well as those from the hearts of patients with congestive heart failure (33).

The observation that digitalis profoundly augments the contractile force of the non-failing heart, without elevating its output, serves to re-emphasize the view that alterations of cardiac output are of little value in predicting changes in myocardial contractility (28). The effects of norepinephrine on the human heart are somewhat analogous, since this drug increases myocardial contractile force (20) without raising cardiac output (34). In the case of digitalis, it is possible that the actions of the drug on the peripheral circulation may be responsible for the discrepancy between the changes in cardiac output and in myocardial contractile force. In other studies in dogs carried out in this laboratory (35), digitalization resulted in an augmentation of intravascular blood volume and a decline in the venous return to the extracorporeal circuit. Similarly, Cotten and Stopp (27) observed in dogs that the decline in cardiac output following digitalization was associated with a fall in left atrial pressure. However, when the left atrial pressure was held constant by means of blood infusion, digitalis resulted in an elevation of cardiac output. It would appear likely that, in the dog, digitalis results in constriction of the hepatic venous sphincter with trapping of blood in the splanchnic bed and elevation of portal venous pressure. The resultant decline in ventricular filling pressure prevents the increased contractility from expressing itself as an increased cardiac output. It is possible that a similar mechanism operates in the human subject without heart failure and accounts for the dissociation between myocardial contractile force and cardiac output. Indeed, the studies of Baschieri, Ricci, Mazzuoli and Vassalle (36) have shown that, in man, digitalis elevates hepatic vein wedge pressure and lowers splanchnic blood flow, thus producing a substantial augmentation of hepatic vascular resistance.

In view of the positive inotropic effect of digitalis on the non-failing heart, the fear of cardiac depression when these drugs are used "prophylactically" (15, 16, 37) would not seem to be warranted. Thus, the exhibition of cardiac glycosides does not appear to be contraindicated in patients without overt heart failure in whom the development of heart failure is feared because of the superimposition of an excessive hemodynamic burden resulting from an acute infection or surgical procedure. Indeed, the demonstration in the present study of the substantial augmentation of contractile force provided by digitalis has led to the establishment of a policy at the National Heart Institute to digitalize all patients prior to intracardiac surgery.

The administration of a drug to a patient on cardiopulmonary bypass makes it possible to determine its direct effects on the systemic vascular resistance. The hemodynamic dissociation of the heart from the peripheral circulation facilitates study of the latter, since any changes in arterial pressure which occur while the systemic perfusion rate is held constant reflect changes in the systemic vascular resistance. Although it has long been suspected that digitalis produces systemic vasoconstriction (38, 39) the powerful cardiac effects of this drug made it difficult to interpret precisely the changes in arterial blood pressure. In an earlier study carried out on dogs on cardiopulmonary bypass, it was demonstrated that digitalis glycosides have a direct pressor effect (40). In the present investigation these observations were extended to and confirmed in man.

SUMMARY

The direct effects of two digitalis preparations on the contractile force of the heart were studied by means of the Walton-Brodie strain gage arch in 21 patients who had never experienced congestive heart failure. Fourteen patients with either atrial septal defect or pulmonic stenosis, studied during cardiopulmonary bypass, were given 0.5 to 1.5 mg of acetylstrophanthidin, a dose which averaged 0.026 mg per kg body weight. This resulted in an increase in contractile force which ranged from 16 to 327 per cent and averaged 89 per cent of the contractile force measurement prior to digitalization. In three patients who received an average dose of 0.022 mg per kg of lanatoside C during cardiopulmonary bypass, the right ventricular contractile force increased by an average of 31 per cent of control levels. In an attempt to study the response of a ventricle which had not been subjected to an abnormal hemodynamic burden for a prolonged period of time, left ventricular

contractile force was recorded immediately after mitral valvulotomy in three patients. Acetylstrophanthidin increased contractile force an average of 24 per cent of the control levels. The administration of the digitalis preparations to patients on cardiopulmonary bypass at a constant perfusion rate permitted study of the direct effects of these drugs on systemic vascular resistance. Acetylstrophanthidin resulted in a brief increase in systemic vascular resistance which averaged 23 per cent and lanatoside C resulted in an average increase of 14 per cent of the control levels. It is concluded that these digitalis preparations augment the contractile force of the non-failing human heart and constrict the systemic vascular bed.

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QUINIDINE: ARTERIAL, VENOUS, CORONARY SINUS AND MYOCARDIAL CONCENTRATIONS *

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Most of the current knowledge of relationships between blood levels of quinidine and therapeutic or toxic effects were ascertained by determining peripheral venous levels following oral administration. After single oral administration of quinidine sulfate to patients with atrial fibrillation, Wegria and Boyle (1) observed that the intensity of the cardiac effect of the drug and its plasma concentration were grossly parallel but not parallel in a strictly quantitative manner. Discrepancies were found between the intensity of cardiac effect and quinidine plasma concentration, the plasma level of the drug decreasing faster than the intensity of its cardiac effect. Some investigators utilize quinidine concentration of venous blood as a guide in determining optimal therapy (2–6). Wegria and Boyle (1) reported no significant difference between the arterial and venous concentration of quinidine a few hours after oral administration. Higher levels were found in the myocardium than in the peripheral venous blood. A recent publication (7) contains a review of the pertinent literature on the subject. In spite of the clinical practice of administering quinidine intravenously to patients with certain life-threatening arrhythmias for immediate therapeutic effects, there is a paucity of data relative to blood levels and these are limited to venous blood (4, 8).

In the present study of the cardiovascular effects of quinidine given intravenously, levels were determined in specimens obtained from a peripheral vein, artery, coronary sinus and myocardium. Quinidine given intravenously reaches the heart directly, thus bypassing the gastrointestinal tract and liver. We attempted to determine whether a gradient could be demonstrated across the myocardium following such administration.

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METHODS

Quinidine gluconate was injected within 1 minute into a cannulated foreleg vein of mongrel dogs anesthetized with sodium pentobarbital. The cannula was kept patent by a drip of heparinized normal saline which was used to flush the injected quinidine into the circulation. Cannulas were inserted into the femoral artery and femoral vein of opposite hind legs for a distance of 5 cm to permit proper collection of specimens. Following cannulation of the opposite femoral artery, the blood pressure was recorded constantly with a Sanborn water manometer or Statham strain gage. Electrocardiograms were continuously recorded. The chest was opened in the right third or fourth intercostal space and a polyethylene tube, small enough not to obstruct the coronary sinus, was inserted into the right atrial appendage and guided into the coronary sinus. After insertion, the tube was sutured to the right atrial wall at a distance from the coronary sinus. The cannula was initially kept patent by a slow drip of heparinized normal saline. In later studies, coronary sinus blood was often permitted to drip constantly. With the help of 3 observers, specimens were withdrawn simultaneously from the femoral vein, femoral artery and coronary sinus at 1, 3, 5, 10, 15 and 30-minute intervals following injection. The collection of samples was completed within 10 seconds. In some instances, the effects of repeated and prolonged injections were determined. In 5 dogs, ventricular biopsies were obtained at varying intervals following the injection of quinidine.

Quinidine levels were determined on whole blood samples by measuring the fluorescence of trichloroacetic acid filtrates, employing the principles of the Linenthal, Ulick and Patterson (9, 10) modification of the Brodie, Udenfriend and Baer technique (11, 12). In each animal, blood samples obtained prior to the administration of quinidine were used as blanks. Minimal to no fluorescence was detected. Initially, an Aminco-Bowman spectrophotofluorometer was employed at an activation wave length of 360 mμ and an emission wave length of 450 mμ. Subsequently, a photofluorometer (Electronic model 12C, Coleman Instrument Co.) was used with a primary and a secondary filter, Corning nos. 5874 and 3389-4308.

A solution of quinidine gluconate containing 100 mg per 100 ml as quinidine base was prepared. One-tenth milliliter of this solution was added to various volumes of blood so that the final theoretical concentration of quinidine base was from 1 to 20 μg per ml blood. These

bloods were analyzed as blind samples and were reported as differences between blood blank and quinidine sample. The mean recovery was 94 per cent, SE 3.9 per cent. Forty specimens were run in quintuplicate. The mean difference of an individual specimen from the mean value of its group was ± 2.93 per cent, SD 2.57 per cent. To ascertain that the measured fluorescence represented unconjugated quinidine, parallel samples of the same blood were extracted according to standard procedure in 10 dogs (9–12). The results indicated that over the period of observation there had been no significant conjugation of the quinidine.

All findings are expressed in terms of quinidine base.

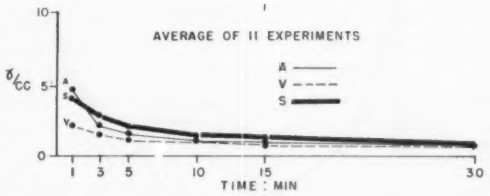

FIG. 1. ARTERIAL, VENOUS AND CORONARY SINUS CONCENTRATIONS OF QUINIDINE BASE (MICROGRAMS PER MILLILITER) AFTER THE RAPID INTRAVENOUS INJECTION OF 4.1 TO 6.0 MG PER KG.

RESULTS

Quinidine gluconate was administered rapidly in amounts varying from 2.0 to 15.0 mg per kg body weight.

A. Dosage levels of 2.0 to 4.0 mg per kg body weight. Four dogs in this group received one injection and two received a second similar dose 15 to 30 minutes after the 30-minute specimen had been obtained. The peak level was reached in the 1-minute arterial specimen with a range of 1.1 to 4.7 μg per ml. The levels then fell abruptly in the first few minutes with plateaus obtained within 5 to 10 minutes. The arterial, venous and coronary sinus levels were essentially similar by 10 minutes with a range from 0.2 to 1.7 μg per ml at 30 minutes. The venous level of quinidine was somewhat lower than the arterial and coronary sinus specimens for the first 5 minutes. There was no significant difference between the arterial and coronary sinus levels. With the second injection, there was an increase in the early levels of quinidine as compared with those following the first injection. The peak arterial level was 5.3 μg per ml and the 30-minute level 2.2 μg per ml. In the subsequent samples, no significant difference was demonstrated as compared with the first injection except for slightly higher levels.

B. Dosage levels of 4.1 to 6.0 mg per kg body weight (*Figure 1*). The 11 dogs in this group received an initial injection of 4.1 to 6.0 mg per kg body weight, and 6 received a similar dose repeated 15 to 30 minutes after the 30-minute specimen was obtained. The peak levels were found in the 1-minute arterial specimens and varied from 2.1 to 12.0 μg per ml. The levels fell abruptly to approximately one-half the peak concentration in the first 5 minutes with a plateau obtained in 10 minutes and little change noted by 30 minutes. The arterial, venous and coronary sinus levels were similar in 10 minutes for any one dog with the levels varying from 0.3 to 3.8 μg per ml by 30 minutes. In six dogs, the quinidine level in the coronary sinus specimen was greater than in the arterial or venous specimens at 3 and 5 minutes. The concentration of quinidine in the venous specimen was consistently lower than that in the arterial or coronary sinus specimens for the first 3 to 5 minutes. In the six dogs which received a second injection of quinidine, the peak arterial level was 2.7 to 12 μg per ml with a 30-minute level of 0.3 to 4.2 μg per ml. In two of these dogs, the concentration of quinidine in the coronary sinus was greater than in the arterial sample obtained at 1 and 3 or 3 and 5 minutes. Each of these dogs showed similar results following the initial injections.

C. Dosage levels of 6.1 to 10.0 mg per kg body weight. Eleven dogs received 6.1 to 10.0 mg of quinidine base per kg body weight as an initial injection. The peak arterial level ranged from 4.6 to 22.2 μg per ml. The levels fell abruptly in 3 minutes with a plateau obtained within 15 to 30 minutes. The arterial, venous and coronary sinus levels in any one dog were similar in 30 minutes, with the levels ranging from 0.5 to 5.7 μg per ml. In eight dogs, the quinidine blood levels were greater in the coronary sinus than in the artery or vein in the 3 to 10-minute specimens. Seven dogs received a second injection of quinidine 15 to 30 minutes after the last 30-minute specimen had been obtained. In three of these dogs, the quinidine level in the coronary sinus blood was greater than in the artery or vein in the 1 and 3 or 3 and 5-minute specimens. The peak arterial level ranged from 0.8 to 14.3 μg per ml and the residual

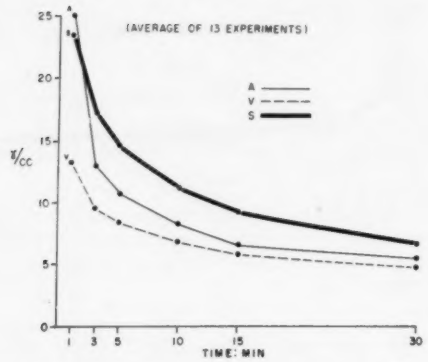

FIG. 2. ARTERIAL, VENOUS AND CORONARY SINUS CONCENTRATIONS OF QUINIDINE BASE (MICROGRAMS PER MILLILITER) AFTER THE RAPID INTRAVENOUS INJECTION OF 15.0 MG PER KG. The concentration of quinidine in the coronary sinus is greater than in the arterial and venous specimens in the 3 to 15-minute samples.

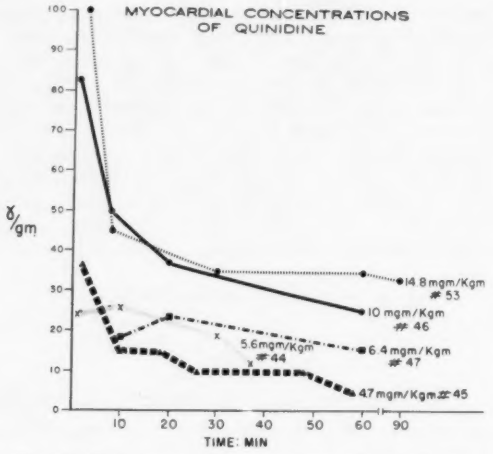

FIG. 3. THE MYOCARDIAL CONCENTRATIONS OF QUINIDINE FOLLOWING VARIOUS INTRAVENOUSLY INJECTED AMOUNTS. The concentration varies directly with the amount injected.

levels at 30 minutes were 1.1 to 5.7 μg per ml. The rapid fall within a few minutes was similar to that observed following the initial injection.

D. Dosage levels of 15.0 mg per kg body weight. Thirteen dogs received 15.0 mg per kg as an initial injection (Figure 2). The peak arterial levels ranged from 11.5 to 59.0 μg per ml. The levels fell abruptly in 3 to 5 minutes with a plateau appearing by 10 to 15 minutes in most instances. In 30 minutes, the arterial, venous and coronary sinus concentrations approached one another; the levels varied from 2.3 to 10.5 μg per ml. In each instance the quinidine level was greater in the coronary sinus blood of the 3-minute specimen and this usually persisted for 10 to 15 minutes. The 1-minute coronary sinus sample showed a higher concentration of quinidine than the arterial specimen in 8 of the 13 dogs. Eight of the dogs received a subsequent repeat injection of 15.0 mg of quinidine base per kg. The peak arterial level ranged from 18.0 to 54.0 μg per ml with 30-minute residual levels of 6.4 to 16.0 μg per ml. In seven trials the 3-minute level of quinidine was highest in the coronary sinus. In the remaining dog, the 5-minute coronary sinus concentration of quinidine was highest. In five studies the 1-minute coronary sinus specimen showed a higher concentration of quinidine than the arterial sample.

E. Myocardial concentrations. Five dogs received 4.7 to 14.8 mg quinidine per kg intravenously (Figure 3). In addition to arterial, venous and coronary sinus samples, biopsies were obtained from the ventricular myocardium at varying intervals up to 90 minutes. The myocardial concentrations of quinidine were four- to tenfold

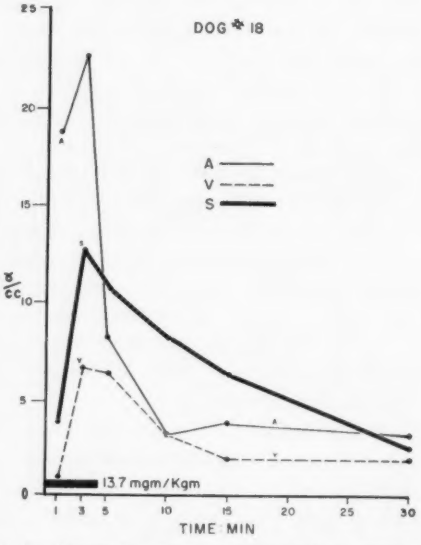

FIG. 4. ARTERIAL, VENOUS AND CORONARY SINUS CONCENTRATIONS FOLLOWING THE INJECTION OF 13.7 MG PER KG OVER A 4.5-MINUTE PERIOD. The concentration of quinidine increases rapidly during the period of injection and then decreases after the injection is stopped. The coronary sinus concentration is greater than the arterial and venous samples until the 20-minute specimen.

higher than the corresponding blood specimens. Three to 6 per cent of the injected amount was initially localized in the myocardium. The myocardial concentrations varied directly with the amount of quinidine injected.

F. Blood pressure. Within 30 to 60 seconds after the injection of 5 or more mg per kg, the blood pressure fell abruptly to two-thirds or less of the control value. A less marked drop usually occurred with smaller injections. The duration of reduced pressure persisted for varying periods depending upon the dosage, and usually for at least 15 minutes. Vasopressors such as Levophed and Aramine effectively prevented this drop in pressure.

G. Prolonged injection. When quinidine is given more slowly, there is a gradual rise in the arterial, venous and coronary sinus concentrations until the injection is discontinued (Figure 4). This results in a more rapid decline in the arterial concentration as compared with the coronary sinus. The level of quinidine in the coronary sinus exceeds the arterial specimen for several minutes. This was observed in three dogs given 11.0 to 14.0 mg per kg over a period of 5 minutes.

DISCUSSION

The intravenous administration of quinidine is usually reserved for emergency use in those patients with certain life-threatening arrhythmias in whom a prompt therapeutic result is required. Most of the available data pertaining to quinidine assay has been obtained after oral, intramuscular or rectal administration (2–4, 9, 13–19). Wegria and Boyle (1) reported no significant differences between the arterial and peripheral venous specimens obtained a few hours after oral administration. Several dogs were sacrificed at hourly intervals following orally administered quinidine. Maximal plasma and tissue quinidine concentrations were reached within 3 hours after ingestion of the drug. Myocardial levels were higher than those of peripheral venous blood. The concentration of quinidine in the left ventricle was 148.6 mg per kg, and in the plasma, 12.5 mg per L 3 hours after 50 mg per kg of quinidine sulfate was given orally. A marked difference persisted up to the final determination at 14 hours.

Sokolow (4) reported that intravenous administration in humans produced high venous levels in a few minutes with the peak subsiding in 5 to 10 minutes. Weisman (20) noted that with a single intravenous dose, less than 6 per cent remained in the blood after 7 minutes. Weiss and Hatcher (21) showed in cats that 95 per cent of an intravenous dose was cleared from the blood in 5 minutes. Kelsey, Oldham and Geiling (22) observed myocardial concentrations of quinidine six times greater than in blood in the rabbit 10 minutes after intravenous injection.

To our knowledge, this is the first attempt to measure and demonstrate quinidine arteriovenous and coronary-sinus arterial differences after intravenous injection of quinidine. There was a fairly wide variation in the blood levels of each animal in spite of the standardization of dose. The variation in the initial arterial level is probably secondary to the rapid change in concentration that occurs shortly after injection. Variations occur in man after the oral administration of quinidine for therapeutic purposes. With increased doses and repeated injections, the blood levels increased and the same general curves resulted. In each dog, the relationships of arterial, venous and coronary sinus levels persisted as described.

The detection of a transient gradient between the coronary sinus and arterial samples suggests several possible alternative explanations. The concentration in the coronary sinus may be higher because of the technique of collection. Conceivably, the catheter partially blocked the coronary sinus so that the specimen reflected the earlier higher arterial level. To obviate this artifact, various sizes of catheters were employed, and similar results were obtained. Moreover, the catheter remained patent in several dogs during the continuous collection of blood. The results were similar whether this technique or a constant slow perfusion with heparinized saline was used. Every effort was made to obtain simultaneously collected specimens.

The concentration of quinidine in the myocardium far exceeded that of the blood. A tentative explanation for the higher concentration in the coronary sinus is based upon the hypothesis that the quinidine is initially bound or pooled in the myocardium and is subsequently washed out by the perfusing blood. The higher coronary sinus concentration may thus represent a washing

out of this bound or pooled quinidine. Preliminary studies indicate that appreciable amounts of quinidine are recovered in the coronary sinus specimens after perfusion of the coronary arteries with saline or whole blood. Quinidine is thus washed out from the myocardium by the perfusing fluid. Weiss and Hatcher (21) interpreted their data as showing that the capillaries of certain organs fix quinidine loosely initially and that much of the fixed drug can be removed by perfusion. Chen and Geiling (23) found that the capillaries of the lungs may be the site of absorption for removal of quinine from the circulation.

Following the intravenous administration of large doses of quinidine, hypotension results uniformly (24, 25). Conceivably, the transient higher concentration of quinidine in the coronary sinus as compared with the arterial blood may be associated with the subsequent hypotension that might promote pooling in the myocardium. Preliminary studies in five dogs indicate that when hypotension is prevented by vasopressor substances, the coronary sinus concentration continues to exceed the arterial levels from the 3 to 5 or 10-minute period. Another factor to be considered is that altered coronary flow may be related to varying quinidine content in the coronary sinus blood, since only limited amounts of quinidine may be released to the perfusing blood in any given period of time. The possibility remains that the increased coronary artery perfusion during higher arterial pressures may reduce the concentration of quinidine in the myocardium.

The extremely high myocardial concentration of quinidine occurs shortly after injection, as demonstrated by the extremely high initial peak and the subsequent rapid decline. Apparently the quinidine is bound rapidly in the myocardium after the initial period of perfusion and subsequently declines as the blood continues to perfuse the myocardium. Quinidine is also found in high concentrations in other tissues, but the presence of arteriovenous differences across these tissues has not been determined.

SUMMARY

1. The rapid intravenous administration of quinidine gluconate in doses of 4.1 to 6.0 mg per kg body weight produces a peak arterial level in 1 minute with a rapid decline within 3 to 5 minutes. In three of six dogs, the quinidine level in the coronary sinus transiently exceeded that in arterial blood following the 1-minute specimen.

2. With the injection of higher doses, the quinidine level in the coronary sinus usually exceeded the arterial level after the first minute.

3. The myocardial concentration of quinidine, following examination of biopsy specimens taken serially in the same animal, exceeded the concentration in the blood from four- to tenfold.

4. It is suggested that quinidine is pooled transiently or bound in the myocardium and is subsequently washed into the coronary sinus.

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BLOCKING OF HOG INTRINSIC FACTOR BY HUMAN GASTRIC JUICE AND CERTAIN MUCOPOLYSACCHARIDES, INCLUDING BLOOD GROUP SUBSTANCE *

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Intrinsic factor can bind vitamin B_{12} and simultaneously attach to receptors on liver or small intestine in a calcium-dependent manner (1). There appears to be a stoichiometric relationship between the receptors, intrinsic factor and vitamin B_{12} (2).

This report, previously presented in preliminary form (3), is concerned with blocking of the receptor sites for hog intrinsic factor by human gastric juice and certain mucopolysaccharides, including blood group substance.

MATERIALS AND METHODS

Gastric juice was collected before and after insulin stimulation of fasting patients. A Dreiling tube (4) was used to avoid contamination with saliva and bile.[1] Gastric juices were pooled according to the blood group of the donors, centrifuged, neutralized with NaOH (20 per cent), and frozen.

Saliva was collected from blood group A-secretors and filtered through gauze.

Commercial preparations of blood group A and B substances[2] were obtained from large pools of equine and porcine gastric mucosa after enzymatic digestion and alcohol precipitation.

Degraded blood group A and B polysaccharides ("P-1 fraction") were prepared from the commercial preparations (Merck) by mild acid hydrolysis with dilute HCl, pH 2.0, at 100° C for 2 hours, followed by dialysis for 4 days against 4 changes of tap water. This treatment splits off fucose and certain oligosaccharide side chains, some of which determine blood group specificity, but greatly increases cross reactivity with type XIV antipneumococcal horse serum (5, 6).

Hog intrinsic factor concentrate (HIFC), WES 727,[3] was potent in a daily oral dose of 5 mg as measured by Schilling-type testing (7). It contained 15 to 20 per cent of blood group A activity as determined by the isohemagglutination technique (5). Twenty-five μg of this preparation was almost sufficient to saturate the receptors in a 10 ml aliquot of 10 g of rat liver homogenate suspended in 100 ml of Krebs-Ringer-Tris (KRT) medium (2). Other agents tested were: chondroitin sulfate (General Biochemicals, lot 26752); human chorionic gonadotropin ("Follutein," Squibb); heparin ("Liquaemin sodium," Organon); pneumococcus polysaccharides types I, VII, XIV and XVIII and Friedlander bacillus polysaccharide (obtained from Dr. Ely Perlman, Department of Bacteriology, The Mount Sinai Hospital, N. Y.); dextran, USP, 6 per cent wt/vol in saline (Abbott); hyaluronic acid powder, purity approximately 90 per cent (prepared by Dr. Boaz and provided by Dr. M. Horowitz, Department of Gastroenterology, The Mount Sinai Hospital, N. Y.); radioactive Co^{60}-vitamin B_{12}[3] diluted to 10^{-6} g per ml, specific activity 1 μc per μg, which re-

TABLE I

Inhibition of hog intrinsic factor concentrate (HIFC) enhancement of liver homogenate uptake of Co^{60}-B_{12} by A, B or O pooled human gastric juice (HGJ) or A-secretor saliva *

First incubation Added agent	Second incubation Added agent	Counts/minute	% Inhibition
Experiment A			
25 μg HIFC plus:			
1 ml 0.9% NaCl	Co^{60}-B_{12}	2,575 ± 261	
1 ml A—HGJ	Co^{60}-B_{12}	612 ± 76	83
1 ml B—HGJ	Co^{60}-B_{12}	574 ± 36	84
1 ml O—HGJ	Co^{60}-B_{12}	395 ± 63	89
Experiment B			
25 μg HIFC plus:			
1 ml 0.9% NaCl	Co^{60}-B_{12}	3,162 ± 182	
1 ml A-secretor saliva	Co^{60}-B_{12}	615 ± 50	81

* Vitamin B_{12} content of pooled HGJ and A-secretor saliva [*Euglena gracilis*, Z strain (8); assay kindly performed by Dr. Herman Baker]. A-HGJ = 76 $\mu\mu$g/ml; B-HGJ = 35 $\mu\mu$g/ml; O-HGJ = 66 $\mu\mu$g/ml; A-secretor saliva = 40 $\mu\mu$g/ml. Approximate concentration of blood group substance in HGJ by iso-hemagglutination technique (5): A-HGJ = 70 μg/ml; B-HGJ = 30 μg/ml.

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TABLE II

Inhibition of HIFC enhancement of liver uptake of Co^{60}-B_{12} by purified A and B blood group polysaccharides

First incubation Added agent	Second incubation Added agent	Third incubation Added agent	Counts/minute	% Inhibition
Experiment A				
25 μg HIFC plus:				
0.9% NaCl	Co^{60}-B_{12}		2,876 ± 18	
25 μg A-bgp	Co^{60}-B_{12}		1,073 ± 25	63
25 μg B-bgp	Co^{60}-B_{12}		2,027 ± 16	31
Experiment B				
0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	2,733 ± 264	
25 μg A-bgp	25 μg HIFC	Co^{60}-B_{12}	461 ± 14	83
25 μg B-bgp	25 μg HIFC	Co^{60}-B_{12}	1,169 ± 25	57

corded approximately 1 cpm per μμg in our well-type scintillation crystal.

Two, three or four sequential incubations in KRT, each followed by three washes, were performed in two ways: *1)* the agent under study was incubated with liver homogenate *prior* to the addition of HIFC and, *2)* the agent under study and HIFC were mixed together and then incubated with liver homogenate. Otherwise, the protocol was as previously outlined (*2*). After final incubation with Co^{60}-B_{12} the radioactivity retained in the washed homogenate was assayed. All samples were run in duplicate. In the tables, the arithmetic mean of the duplicate samples is recorded, ± the actual deviation from the mean.

RESULTS

The tables record the liver homogenate uptake of Co^{60}-B_{12} after pre-incubation with HIFC and the decrease of that uptake recorded as "per cent inhibition" due to the agents tested.

Pooled human gastric juice (HGJ) from A, B, and O individuals and saliva from a blood group A-secretor each diminish the effect of HIFC on the uptake of Co^{60}-B_{12} by rat liver homogenate (Table I).

Purified blood group A or B polysaccharides (bgp), either mixed with HIFC in equal concentration and then incubated with liver homogenate (Table II, Experiment A), or added before incubation with HIFC (Experiment B), reduced the effect of HIFC. This reduction was greater when the homogenate was exposed to bgp before exposure to HIFC.

Pooled human gastric juice from blood group A donors (A-HGJ) retains its ability to block the effect of HIFC after heating or dialysis (Table III).

This inhibitory effect of A-HGJ may be almost eliminated by suspending liver homogenate previously incubated with A-HGJ in ethylenediamene-

TABLE III

Inhibition of HIFC effect on uptake of Co^{60}-B_{12} by heated or dialyzed pooled human gastric juice

First incubation Added agent	Second incubation Added agent	Counts/minute	% Inhibition
Experiment A			
Heated* A-HGJ plus 25 μg HIFC	Co^{60}-B_{12}	95 ± 15	95
Unheated A-HGJ plus 25 μg HIFC	Co^{60}-B_{12}	63 ± 1	97
0.9% NaCl plus 25 μg HIFC	Co^{60}-B_{12}	1,928 ± 14	
Experiment B			
Nondialyzed A-HGJ plus 25 μg HIFC	Co^{60}-B_{12}	905 ± 67	76
0.9% NaCl plus 25 μg HIFC	Co^{60}-B_{12}	3,764 ± 152	
Experiment C			
Dialyzed† A-HGJ plus 25 μg HIFC	Co^{60}-B_{12}	747 ± 0	76
0.9% NaCl plus 25 μg HIFC	Co^{60}-B_{12}	3,095 ± 0	

* HGJ heated in boiling water bath at 100° C for 20 minutes.
† HGJ dialyzed at 4°C for 22 hours against 2 changes of 4 L of distilled water.

TABLE IV

Reversibility with incubation in EDTA or 0.9 per cent NaCl of the inhibitory effect of A blood group-containing pooled gastric juice on the effect of HIFC on liver homogenate Co^{60}-B_{12} uptake

First incubation Medium, KRT Added agent	Second incubation Suspended in	Third incubation Medium, KRT Added agent	Fourth incubation Medium, KRT Added agent	Counts/minute
1 ml A-HGJ	10 ml 0.015 EDTA	25 μg HIFC	Co^{60}-B_{12}	2,539 ± 93
25 μg HIFC	10 ml 0.015 EDTA	NaCl	Co^{60}-B_{12}	46 ± 11
NaCl	10 ml 0.015 EDTA	25 μg HIFC	Co^{60}-B_{12}	3,187 ± 28
1 ml A-HGJ	10 ml 0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	2,902 ± 364
25 μg HIFC	10 ml 0.9% NaCl	NaCl	Co^{60}-B_{12}	1,423 ± 156
25 μg HIFC	10 ml 0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	3,525 ± 21

tetraacetate (EDTA) or 0.9 per cent NaCl at 37° (Table IV).

When A and B bgp were incubated with liver homogenate without Ca^{++} in the medium (KRT, where Ca^{++} was replaced by an equivalent millimolar concentration of Na^{+}) and HIFC was added to a subsequent incubation, the HIFC effect was minimally depressed (Table V). Conversely, with Ca^{++} present in the medium during the pre-incubation with bgp, the effect of subsequently added HIFC was markedly diminished (Table V).

When the homogenate was incubated in either EDTA or 0.9 per cent NaCl after incubation with A or B-bgp or pneumococcus polysaccharide type XIV, there was no inhibition of the effect of subsequently added HIFC (Table VI). The effect of HIFC itself, however, was still marked after incubation in 0.9 per cent NaCl but not after incubation in EDTA (Table VI).

Some dialyzable sugars (fucose, certain oligosaccharide side chains) are released from blood group polysaccharides by mild acid hydrolysis.

TABLE V

Calcium-dependence of the effect of blood group substance on liver homogenate Co^{60}-B_{12} uptake after incubation with HIFC

First incubation Medium, KRT Added agent	Second incubation Medium, KRT Added agent	Counts/minute
100 μg A-bgp	25 μg HIFC	324 ± 1
100 μg B-bgp	25 μg HIFC	678 ± 85
0.9% NaCl	25 μg HIFC	2,836 ± 36
Medium, KRT(-Ca)		
100 μg A-bgp	25 μg HIFC	2,660 ± 30
100 μg B-bgp	25 μg HIFC	2,514 ± 102
0.9% NaCl	25 μg HIFC	3,963 ± 31

The nondialyzable residue (P-1 fraction) (5, 6) was resuspended in 0.9 per cent NaCl and tested (as "degraded bgp"). Less degraded bgp was needed to obtain the same "per cent inhibition" of HIFC than was obtained with intact bgp (Table VII). The decrease in Co^{60}-B_{12} uptake was proportional to the increased amount of the "inhibitor."

Table VIII records the inhibition by 25 μg aliquots of 16 different polysaccharides of the HIFC effect on the Co^{60}-B_{12} uptake of rat liver homogenate. A moderate inhibitory effect of relatively large quantities of Cohn fractions III and IV, and haptoglobin and gastric juice from patients with pernicious anemia, has also been demonstrated.

DISCUSSION

The ability of hog intrinsic factor concentrate to enhance Co^{60}-B_{12} uptake by rat liver homogenate was inhibited *in vitro* by normal human gastric juice from A, B, or O donors, or saliva from an A-secretor. The inhibitory effect remains after heating or dialysis of blood group A human gastric juice. Inhibition is greater when the agent under study is pre-incubated with the liver homogenate before addition of HIFC rather than when it is mixed with HIFC and then added to the homogenate.

This suggests that the inhibitory agents act by attaching to the liver "receptors" for intrinsic factor, thereby blocking attachment of intrinsic factor to the receptors.

The blocking of the intrinsic factor receptors by the various agents studied appears to be calcium-dependent, and reversible by removing Ca^{++} from the system. The various "inhibitors" did not damage the integrity of the liver "receptors"

TABLE VI

Reversibility with incubation in EDTA or 0.9 per cent NaCl of the inhibition by certain polysaccharides of the effect of HIFC on liver homogenate Co^{60}-B_{12} uptake

First incubation Medium, KRT Added agent	Second incubation Medium	Third incubation Medium, KRT Added agent	Fourth incubation Medium, KRT Added agent	Counts/minute
25 μg A-bgp	10 ml 0.015 M EDTA	25 μg HIFC	Co^{60}-B_{12}	2,627 ± 127
25 μg B-bgp	10 ml 0.015 M EDTA	25 μg HIFC	Co^{60}-B_{12}	2,921 ± 353
25 μg PnS XIV*	10 ml 0.015 M EDTA	25 μg HIFC	Co^{60}-B_{12}	2,752 ± 363
1 ml 0.9% NaCl	10 ml 0.015 M EDTA	25 μg HIFC	Co^{60}-B_{12}	2,468 ± 12
25 μg HIFC	10 ml 0.015 M EDTA	0.9% NaCl	Co^{60}-B_{12}	224 ± 12
25 μg A-bgp	10 ml 0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	2,320 ± 72
25 μg B-bgp	10 ml 0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	2,765 ± 173
25 μg PnS XIV	10 ml 0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	2,816 ± 113
1 ml 0.9% NaCl	10 ml 0.9% NaCl	25 μg HIFC	Co^{60}-B_{12}	2,944 ± 120
25 μg HIFC	10 ml 0.9% NaCl	0.9% NaCl	Co^{60}-B_{12}	1,022 ± 72

* Pneumococcus polysaccharide type XIV.

as manifested by the fact that the receptors remained capable of again binding HIFC after removal of the inhibitor. The relative ease of removal of polysaccharide inhibitors as compared with HIFC, on incubation in 0.9 per cent NaCl, suggests a lesser specificity of these agents than of HIFC for liver receptors.

In accordance with the concept of molecular complementarity as the basis of specificity (9), the minimal blocking effect of certain polysaccharides and the strong blocking effect of others suggest that there is a specific moiety of polysaccharide nature involved in the binding of intrinsic factor to liver receptors.

It is possible that this moiety of intrinsic factor consists mainly of terminal β-galactosyl groups. This is suggested by the findings that the most active "blockers" are P-1 fractions prepared from blood group substances and type XIV pneumococcus polysaccharide, all of which react strongly with type XIV antipneumococcal horse serum (5, 6), the reaction appearing related to the presence of terminal β-galactosyl groups (10). The blood group substances themselves are somewhat less effective as blockers than their P-1 fractions, and other pneumococcus polysaccharides studied are less effective than type XIV. The postulated β-galactosyl end-group configuration may not be identical for intrinsic factor derived from different species.

These studies leave intact the concept (11) that the ability to bind to both receptor and B_{12}

TABLE VII

Inhibition of HIFC activity when mixed with intact or degraded blood group polysaccharides

Agent mixed with 25 μg HIFC	Amount*	Inhibition
	μg	%
Intact A-bgp	25	74
Intact B-bgp	25	55
Degraded A-bgp ("P-1 fraction")	25	90
Degraded A-bgp ("P-1 fraction")	10	75
Degraded A-bgp ("P-1 fraction")	5	56
Degraded B-bgp ("P-1 fraction")	25	85
Degraded B-bgp ("P-1 fraction")	10	65
Degraded B-bgp ("P-1 fraction")	5	54

* Amounts of degraded bgp are stated as the weight of intact bgp from which they were derived.

TABLE VIII

Inhibition of enhanced liver homogenate Co^{60}-B_{12} uptake due to HIFC by polysaccharides

Agent*	% Inhibition
A-bgp	74
B-bgp	55
Degraded A-bgp ("P-1 fraction")	90
Degraded B-bgp ("P-1 fraction")	75
Pneum. polysaccharide Type XIV	65
Pneum. polysaccharide Type I	11
Pneum. polysaccharide Type XVIII	8
Pneum. polysaccharide Type VII	30
Pneum. polysaccharide "C"	4
Hyaluronic acid	5
Bacillus polysaccharide Friedlander	8
Human chorionic gonadotropin	6
Heparin	2
Chondroitin sulfate	13
Dextran	1
Agar ("Bacto-Agar," Difco; purified†)	5

* Twenty-five μg of agent and of HIFC mixed prior to incubation.

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simultaneously may be unique to intrinsic factor. Preliminary experiments with aqueous extracts (12) of consecutive segments of rat small intestine indicate that such extracts may also act to block the intrinsic factor receptor sites on rat liver homogenate. This blocking action diminishes progressively in the more distally located segments and may be related to the presence of decreased quantities of polysaccharides. It has been demonstrated (13) that the average quantity of blood group substance in human intestine is less in extracts made from more distal segments of small intestine. Thus, the greater vitamin B_{12} absorption from ileum (14) than from duodenum and jejunum may be related to a lesser quantity of intestinal receptor blocking materials in the lower small intestine.

The fact that HGJ does not enhance Co^{60}-B_{12} uptake by liver homogenate as much as does HIFC suggests that the former may contain a variable amount of substances able to attach to receptors and thereby block intrinsic factor but unable then to bind B_{12}. One such substance may be degraded intrinsic factor. This possibility is strengthened by the observation that HGJ, degraded by heat, functions as a blocking agent. The blocking action of degraded or undegraded blood group substance and other compounds secreted into the alimentary tract may constitute the major difficulty in the *in vitro* assay of intrinsic factor in human gastric juice, and may in part explain why such assay is generally reliable only in achlorhydric patients (15), who would have reduced ability to degrade blood group substances and intrinsic factor.

Refractoriness of some patients with pernicious anemia to HIFC therapy has been ascribed to an antigen-antibody reaction; it was suggested (15) that a possible antigen might be blood group A substance, or another contaminant in HIFC, rather than intrinsic factor itself. The present studies indicate that the antigen could be a degradation product of HIFC or of blood group substance, or even type XIV pneumococcus polysaccharide. In this connection it should be noted that P-1 fractions prepared from A substance *are antigenic in A individuals*. Antibody (precipitin) to such fractions does not agglutinate erythrocytes or sensitize red cells to render them agglutinable by Coombs serum (6).

However, refractoriness to HIFC may not be due to antigenicity of hog intrinsic factor since it has been demonstrated that sera of some patients with pernicious anemia, who have never received HIFC, inhibit the activity of HIFC (16). Another factor to consider in the "refractory" case is that the oral administration of a poor B_{12}-intrinsic factor product may produce only a minimal hemopoietic response (17).

The question legitimately arises as to the general applicability of data obtained in a heterologous system, such as the present one (HIFC with rat liver homogenate). In this connection the following points should be made. *1*) rat intrinsic factor has been successfully used in place of HIFC in our experimental protocol (*2*). *2*) Species specificity does not appear to play a role in the phase of intrinsic factor action under discussion here. Recent evidence suggests that species specificity is not involved in the attachment of intrinsic factor to receptors, but rather in the subsequent release of vitamin B_{12} from intrinsic factor by a presumed enzyme (12, 18).

These studies may have some relation to the statistical association of pernicious anemia and blood group A, although such relationship is highly speculative (19, 20).

SUMMARY

The enhancing effect of hog intrinsic factor concentrate (HIFC) on Co^{60}-B_{12} uptake by rat liver homogenate receptors may be reduced ("blocked") by blood group substance, degraded blood group substance ("P-1 fractions"), type XIV pneumococcus polysaccharide, human gastric juice (from A, B, or O subjects; undegraded, dialyzed, or heated), and to a lesser extent by other polysaccharides. Blocking is Ca^{++}-dependent and EDTA-reversible, as is the effect of intrinsic factor. In accordance with the concept of molecular complementarity as the basis of specificity, it is suggested that intrinsic factor may have terminal β-galactosyl groups which link to receptors on liver (or small intestine), and that various mucopolysaccharides are effective as blockers of this linkage in proportion to the similarity of their end-group conformation to that of intrinsic factor.

The possibility is raised that human refractoriness to HIFC may be due in part to cross reaction

with antibody to P-1 fractions or type XIV pneumococcus polysaccharide.

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THE DISAPPEARANCE OF 7-H^3-*d*-ALDOSTERONE IN THE PLASMA OF NORMAL SUBJECTS *

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The study of the disappearance of the radioactivity in plasma after the injection of labeled aldosterone should elucidate the transport and metabolism of this steroid in man. In particular this would allow the calculation of the turnover rate in various clinical conditions. This value, together with a concomitant determination of the secretion rate estimated from the specific activity of a urinary metabolite, would enable the mean blood concentration of aldosterone to be calculated. This is particularly important at the present time, as no practical direct method has yet been reported for the analysis of aldosterone in peripheral blood.

After the administration of 16-H^3-aldosterone to one normal subject, the labeled hormone had a half-life in plasma of 20 minutes, as calculated from the change in concentration of radioactivity measured specifically as aldosterone between 2 and 3 hours after the injection (1). This indicated that the rate of metabolism of aldosterone was greater than that of cortisol and corticosterone. The data obtained were not sufficient to give a reliable estimate of volumes of distribution. Also, the low specific activity of aldosterone then available made it necessary to administer 2 μg of hormone. This amount represents about 25 per cent of total body pool of hormone. Peterson reported a corresponding half-life of 0.5 to 0.8 hour in normal subjects after injection of tritiated *d,l*-aldosterone (2). Again in this study large quantities of the hormone were injected. Also it is not known whether the optical isomers of aldosterone are metabolized in an identical manner. The natural hormone is in the *d* form exclusively.

The availability of 7-H^3-*d*-aldosterone of specific activity 20 μc per μg has made it possible to inject 0.1 μg of the hormone (about 1 per cent of the body pool). A more detailed analysis and interpretation of the disappearance curve in plasma has been carried out.

METHODS

4-C^{14}-aldosterone [1] was fractionated by partition column chromatography before use. 7-H^3-aldosterone was prepared as previously described (3). It was stored at a concentration of 11 μc (0.55 μg) per ml in ethanol and checked at regular intervals for radiochemical purity over a period of 2 months. *d,l*-Aldosterone diacetate was prepared by acetylating *d,l*-aldosterone-21-monoacetate.[2] Methylene dichloride was prepared by washing with an equal volume of distilled water and dried over anhydrous sodium sulfate. After filtration, charcoal was added and the solvent decanted and again filtered. It was allowed to stand over potassium hydroxide, refluxed for 1 hour and distilled. It was stored at $-10°$C. Pyridine and acetic anhydride were prepared as previously described (3).

Two-tenths ml of the solution of radioactive aldosterone in ethanol was added to 11 ml physiological saline; 10 ml of this saline solution, containing 2 μc and 0.1 μg 7-H^3-aldosterone was injected into the antecubital vein of female subjects at 8 a.m. Blood was withdrawn from the other arm with a heparinized syringe at various times after injection. The blood was immediately centrifuged and the plasma added to the tube which contained a dry sample of 4-C^{14}-aldosterone [usually 80 dpm (1 μg) per 10 ml plasma]. The plasma was then extracted with 3×1.3 vol of methylene dichloride. The combined solvent extracts were washed once with water (0.3 of the volume of the plasma) and taken to dryness. The mixture was transferred to a smaller flask and acetylated with 1 ml pyridine and 0.5 ml acetic anhydride overnight at room temperature, and taken to dryness. After addition of 8 μg *d,l*-aldosterone diacetate in 1 ml ethanol, the dried residue was transferred to a partition column in 1 ml $+ 2 \times 0.5$ ml mobile phase. The column, 60 cm

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[1] 4-C^{14}-aldosterone was generously donated by Dr. R. Travis and Dr. G. Farrell.

[2] *d,l*-Aldosterone-21-monoacetate kindly supplied by Dr. R. Gaunt, Ciba, Summit, N. J.

long, contained 28 g Celite 545, 14 ml of the stationary phase, water : methanol, 4 : 1, vol : vol and was developed with toluene : Skellysolve C, 2 : 3, vol : vol as the mobile phase; 5-ml fractions were collected. Two-tenths ml from fractions 11 to 18 (counted from the time of application of the extract) was applied separately to no. 2 Whatman paper, immediately sprayed with reagent and detected by soda fluorescence (4). Usually, the aldosterone appeared in fractions 14, 15 and 16. The 3 fractions containing the steroid were pooled, added to quartz vials (Atomic Associates) and taken to dryness in a vacuum oven at 60°C. Ten ml of scintillation fluid (4 g diphenyloxazole plus 100 mg POPOP [3] per liter toluene) was added plus 0.2 ml ethanol. The vials were then assayed for H^3 and C^{14} in an automatic liquid scintillator (Tricarb model 314-DC, Packard Instrument Co.). The samples, together with appropriate background and standard vials, were counted for at least eight 30-minute periods at each high voltage position. Quenching corrections were not necessary for fractions obtained after partition chromatography. The counting rates due to H^3 and C^{14} estimated in this way gave a value for the initial tritium content of the plasma, present specifically as aldosterone (hereafter "radioactive concentrations in plasma after injection of aldosterone" refers to values measured *specifically* as aldosterone) and corrected for losses during the isolation procedure. Accuracy was within 5 per cent (including counting errors) for all samples. The fractions on either side of the aldosterone diacetate peak were also assayed for tritium and C^{14} in 6 experiments. In all cases, the radioactivity was slight. The H^3/C^{14} ratio, when it could be measured, was the same as that in the peak fractions. The recovery of the 4-C^{14}-aldosterone through the isolation procedure in the 3 peak fractions was about 70 per cent. After passing 300 ml of mobile phase through the columns following elution of the steroid and replacing 0.5 g Celite at the top of the column, they could be reused many times. When this procedure was followed there was no deterioration of resolving power, or displacement of the position of the steroid peak or accumulation of radioactivity even after frequent use of the columns.

For some plasma samples (usually taken 22.5 and 30 minutes after injection) 20 per cent of the extract was directly assayed for radioactivity before acetylation. Measurements for H^3 and C^{14} were carried out as previously described, except that quenching corrections were applied by adding internal H^3 and C^{14} standards. For all subjects studied, the total tritium radioactivity in the plasma at 22.5 and 30 minutes after injection was equal to the H^3 measured specifically as aldosterone. It was, therefore, considered that the total tritium radioactivity in the plasma taken 7.5 and 15 minutes after injection was entirely due to aldosterone. The total methylene dichloride extracts from the 7.5 and 15-minute specimens were assayed directly for H^3 and C^{14} without acetylation or chromatography. The quenching correction for H^3 in these crude solutions was about 10 per cent. All samples taken later than this were chromatographed before counting.

Following the injection of 7-H^3-aldosterone, urine was collected for 24 hours and the secretion rate of the hormone estimated from a measurement of the specific activity of aldosterone released from the urinary "3-oxo conjugate" (3).

Three subjects were clinic patients in the department of gynecology. Two were fully recovered and convalescent after pelvic inflammatory disease. One was studied prior to elective hysterectomy for leiomyomata uteri. All three patients were receiving regular diets. They were in the normal range of weight and height, and showed no signs of disturbance of electrolyte metabolism. Two volunteer normal women were studied by a single injection technique over a 3 hour period. The characteristics of their disappearance curves were similar to those of the clinic patients. One normal woman was studied by a constant infusion technique after a priming dose; 1.05 μc 7-H^3-aldosterone was injected instantaneously in 10 ml physiological saline at the start of the experiment. Thirty minutes later, a total of 0.945 μc of the same steroid was given in physiological saline as a constant infusion over a period of 90 minutes at the rate of 0.191 ml (0.0105 μc) per minute. Five ml of plasma was withdrawn 7.5 and 15 minutes after the single injection. These were analyzed without preliminary chromatographic separation and 10 ml of plasma (taken at 30, 105, 120 and 140 minutes after the first injection) was analyzed after chromatography of the diacetate as previously described.

RESULTS

The values for the radioactivity present specifically as aldosterone and fully corrected for recovery, in plasma taken 7.5, 15, 22.5, 30, 50 and 70 minutes after injection of 0.1 μg 7-H^3-aldosterone, are shown in Figures 1, 2 and 3. They

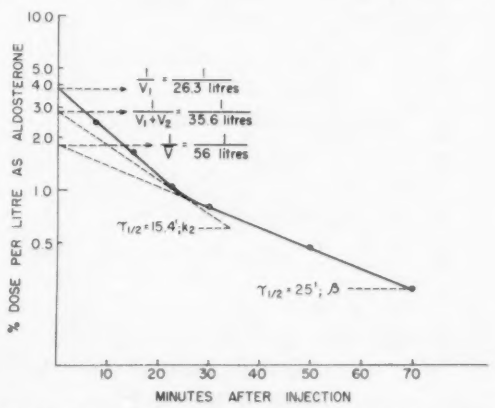

FIG. 1. DISAPPEARANCE OF 7-H^3-ALDOSTERONE IN PLASMA OF NORMAL CLINIC SUBJECT, E. A.

[3] 1,4-*bis*-2-(5 phenyloxazolyl)-benzene.

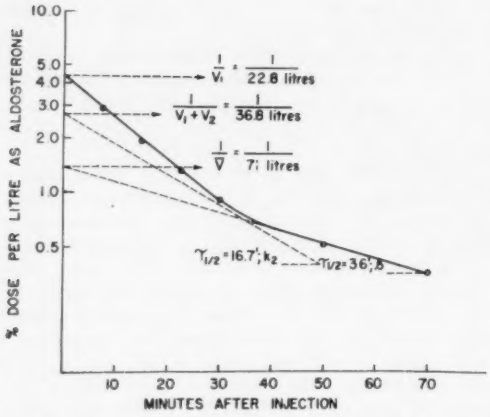

FIG. 2. DISAPPEARANCE OF 7-H³-ALDOSTERONE IN PLASMA OF NORMAL CLINIC SUBJECT, M. C.

are plotted as logarithm of per cent dose injected per liter of plasma against time after injection. These represent disappearance curves for the three clinic patients. For all these subjects, the volumes of plasma taken for analysis were 5 ml at 7.5, 15 and 22.5 minutes, 10 ml at 30 minutes, and 20 ml at 50 and 70 minutes. The disappearance curves obtained in the two volunteers are shown in Figures 4 and 5. The data were obtained as for the other three subjects but, in addition, 50 ml plasma at 120 minutes and 100 ml at 180 minutes were also analyzed.

In all five subjects there was an extremely rapid initial drop in plasma radioactive concentration to about 4 per cent of the injected dose per L of plasma. The curve then became less steep (with a half-life of about 14 minutes) until some 30 minutes after injection. At this time, the plasma concentration was about 1 per cent of the injected dose per L of plasma. The curve then again became less steep in all cases and was a straight line for three of the five subjects. Because of the low concentration of radioactivity in plasma after the injection of a tracer amount of hormone requiring large amounts of blood to be assayed, the slope of the final part of the curve was estimated from plasma samples taken up to 70 minutes after injection in three patients. However, Figures 4 and 5 illustrate that the slope had become constant by that time. Statistical analysis of the data of these two experiments shows no significant difference in the curves between 30 to 70 minutes and 70 to 180 minutes after injection.

The values for the patient who was studied by constant infusion technique after a priming dose

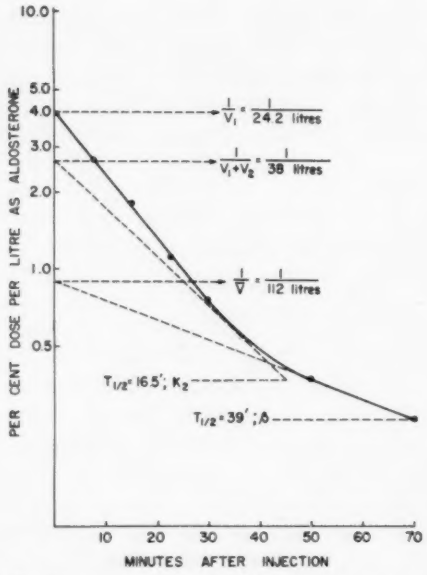

FIG. 3. DISAPPEARANCE OF 7-H³-ALDOSTERONE IN PLASMA OF NORMAL CLINIC SUBJECT, A. A.

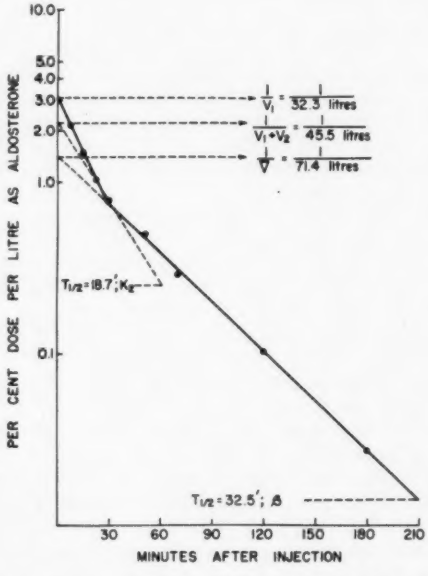

FIG. 4. DISAPPEARANCE OF 7-H³-ALDOSTERONE IN PLASMA OF NORMAL VOLUNTEER, J. D.

are shown in Figure 6. The following values were obtained, expressed as millimicrocuries aldosterone per liter of plasma: 29.9, 15, 9.9, 9.5 and 8.8, for the 7.5, 15, 30, 105 and 120 minute samples, respectively. Therefore, the radioactivity as aldosterone was constant from 30 to 120 minutes after the first injection within the limits of experimental error. The values as radioactive aldosterone 140 minutes after the single injection (20 minutes after the end of the constant infusion) was 6.4 mμc per L plasma.

Calculations. From the characteristics of these disappearance curves the concentration of radioactivity in plasma as a function of time may be expressed as

$$x = Ae^{-\alpha T} + Be^{-\beta T}$$

where x = per cent injected radioactive dose per liter of plasma as aldosterone at time T.

B is the intercept on the ordinate of the extrapolated part of the curve (semilog plot, Figure 7). A + B is the corresponding intercept from the earlier part of the curve. β is the slope of the final part of the curve. $\beta = 1000/T_{\frac{1}{2}}$ (min) (units per day). Both B and β are calculated from the 50′ and 70′ values for Figures 2 and 3, from the 30′, 50′ and 70′ values for Figure 1 and the 30′, 50′, 70′, 120′ and 180′ values for Figures 4 and 5; α is calculated as follows: the latter part of the curve is extrapolated (intercepting the ordinate at B, Fig. 7) and the resulting calculated plasma concentrations subtracted from the measured values. These corrected concentrations (from 0 to 30 minutes) are plotted semilogarithmically and the slope is α. Table I shows the calculated values for A, B, α and β for the five subjects described. Where possible (Figures 1, 4, 5) these were obtained by the method of least squares.

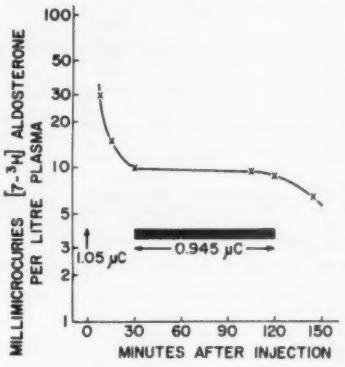

FIG. 6. CONCENTRATION OF 7-H³-ALDOSTERONE IN PLASMA OF NORMAL SUBJECT, Y. A. Priming dose of 1.05 μc followed 30 minutes later by a constant infusion for 90 minutes of a total of 0.945 μc.

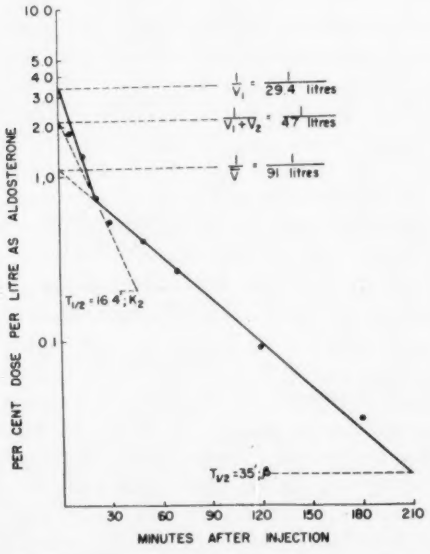

FIG. 5. DISAPPEARANCE OF 7-H³-ALDOSTERONE IN PLASMA OF NORMAL VOLUNTEER, A. M.

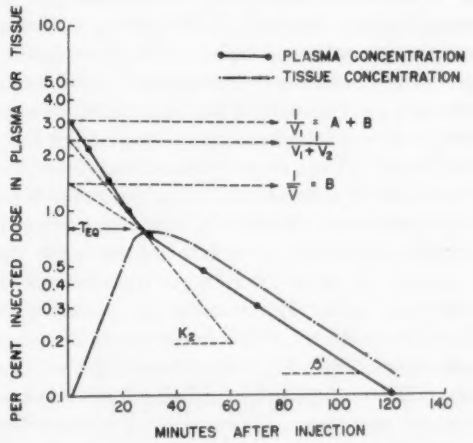

FIG. 7. CONCENTRATION OF RADIOACTIVITY IN INNER POOL (PLASMA) AND OUTER POOL (TISSUE) TO BE EXPECTED FROM MODEL IN FIGURE 8.

TABLE I

Calculated transport and metabolism rate constants and volumes of distribution of aldosterone in five normal women

Subject	A	B	α	β	K_1	K_2	V_1	V_1+V_2	$\bar{V}$
	% dose/L			*units/day*				*liters*	
Patient E. A.	2.0	1.8	143	40	31.4	65	26.3	35.6	55.5
Patient M. C.	3.0	1.4	128	27.8	37.0	60	22.8	36.8	71
Patient A. A.	3.2	0.89	100	25.6	47.3	60.5	24.2	38.0	112
J. D., normal	1.7	1.4	118	30.8	30.0	53.6	32.3	45.5	71
A. M., normal	2.3	1.1	133	28.5	37.0	61.1	29.4	47.0	91
Mean values	2.4	1.3	124	30.5	36.5	60.0	27.0	40.6	80.1

DISCUSSION

Theoretical. A reasonable model for the transport and metabolism of a steroid in man consists of an inner pool which, for steroids weakly bound to plasma proteins, probably includes the plasma and extracellular volumes, and an outer pool (Figure 8). Transport between the spaces can be described by a rate constant K_1. If the liver volume is relatively small, and transport from plasma to metabolic cells proceeds at a reasonable rate, it can be considered as part of the inner pool as regards metabolic events.[4] This seems reasonable in view of the fact that molecules much larger than steroids such as albumin are metabolized in the liver and must be transported into the cells. The over-all metabolism of this pool, total volume V_1, can be described by the rate constant K_2. The total volume of the outer pool is V_2. In the present treatment, metabolism in the outer pool is taken as being negligible. This must be assumed, because analyses in the outer pool are at present impossible to obtain and otherwise, solutions of any mathematical treatment would be indeterminate. However, it has been shown that the liver is efficient in metabolizing aldosterone (5–8). Also Davis (9) has recently shown that the rate of metabolism of aldosterone is very low in the hepatectomized dog. Therefore, this assumption is probably reasonable as a first approach for this hormone. If all radioactivity is measured as aldosterone, then: K_1 = fraction of injected dose per milliliter of plasma transported to the outer pool in unit time; K_2 = fraction of injected dose per milliliter of plasma metabolized in the inner pool per unit time; x = fraction of injected dose per milliliter of plasma at time T; z = fraction of injected dose per milliliter of outer pool at time T.

$$V_1 \frac{dx}{dT} = -V_1K_1x - V_1K_2x + V_1K_1z$$

$$V_2 \frac{dz}{dT} = V_1K_1x - V_1K_1z$$

$$\text{and } x = Ae^{-\alpha T} + Be^{-\beta T}$$

$$z = D(e^{-\alpha T} - e^{-\beta T})$$

$$\text{where } A = \left(\frac{\frac{K_1V_1}{V_2} - \alpha}{\beta - \alpha}\right) \times \frac{1}{V_1};$$

$$B = \left(\frac{\beta - \frac{K_1V_1}{V_2}}{\beta - \alpha}\right) \times \frac{1}{V_1}$$

$$\text{and } D = \left(\frac{K_1}{V_2}\right) \times \frac{1}{\beta - \alpha}$$

$$\frac{K_1V_1 \times K_2}{V_2} = \alpha\beta;$$

$$K_1 + K_2 + \frac{K_1V_1}{V_2} = \alpha + \beta$$

$$\text{and} \qquad K_2 = \frac{\alpha\beta(A + B)}{A\beta + B\alpha};$$

$$V_1 + V_2 = \frac{\beta^2A + B\alpha^2}{(A\beta + B\alpha)^2}$$

$$K_1 = V_2(A\beta + B\alpha).$$

Figure 7 shows some hypothetical radioactive concentrations in plasma and tissue at various times after injection. As has been pointed out by several investigators in other fields (10–14), the radioactive concentration in the outer pool is lower than that in the inner pool for some time after injection. These concentrations then become equal when that in the outer pool is maximal

[4] However, any reversible spread of steroid into liver tissue after the first few minutes after injection would be considered as transport into the outer pool.

(14). From this time on, the radioactive concentration in the outer pool is greater than in the inner. The final slope of the plasma concentration (β) is a result of a combination of the effects of metabolism and transport. It will be a flatter slope than would be expected from the effects of metabolism alone ($\beta < K_2$) because continuous transport occurs from the outer pool into the inner pool. The slope of the curve will only be equal to K_2 when the outer and inner radioactive concentrations are equal (at time T_{Eq}). Similarly, the intercept on the ordinate from the extrapolated later curve (B) gives a volume ($\overline{V}$) ($\overline{V} = 1/B$) which is not the true volume of distribution. This volume multiplied by the radioactive concentration in the plasma will give the total radioactivity as the hormone at any particular time. However, the radioactive concentration in the outer pool is greater than in the inner, therefore the volume $\overline{V}$ is greater than the more meaningful volume of distribution ($V_1 + V_2$). This volume ($V_1 + V_2$) multiplied by the blood concentration of steroid will be equal to the total body content of nonisotopic hormone. $V_1 + V_2$ can be calculated from the expressions given. It is also the reciprocal of the intercept on the ordinate obtained by extrapolating, using the slope K_2, from the radioactive concentration at the equilibrium time as shown in Figure 7. The line in Figures 1–5 and 7 which intercepts the ordinate at $1/(V_1 + V_2)$ and has a slope K_2 has been drawn from the calculated values of $1/(V_1 + V_2)$ and K_2. V_1 is given by $1/(A + B)$. The calculated values of V_1, $V_1 + V_2$, $\overline{V}$, K_1 and K_2 for five subjects are shown in Table I.

General. After a rapid intravenous injection of 4-C^{14}-cortisol into man, the radioactive hormone is distributed extremely quickly into a space of about 6 L (2). After this initial distribution, the radioactivity then appears to move more slowly into a total apparent volume ($\overline{V}$) of about 16 L at 30 minutes after injection. The final slope (β) can then be described by a half-life of about 80 minutes. Peterson has interpreted this curve as indicating a fairly rapid distribution of cortisol into about a 16 L volume and then assumes that this volume is a single compartment in which the hormone has a metabolic half-life of about 80 minutes. In terms of the model previously described, this

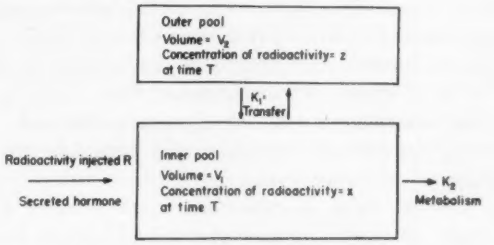

FIG. 8. MODEL DESCRIBING THE METABOLISM AND TRANSPORT OF STEROIDS.

curve could also be interpreted as indicating an extremely rapid distribution into a space of about 6 L (V_1) and then a relatively slow movement into a total volume ($V_1 + V_2$) of about 13 L. The smaller volume (V_1) can be regarded as a single compartment which has a metabolic half-life of about 30 minutes.

In contrast, there is little doubt that the transport and metabolism of aldosterone requires at least a two-compartmental model for interpretation. For aldosterone there is an initial rapid distribution into a space of 20 to 30 L (V_1), which is much greater than the corresponding volume for cortisol. After this there is a slower movement of radioactivity into a total apparent space of 50 to 120 L ($\overline{V}$) or a calculated volume ($V_1 + V_2$) of about 40 L. Whatever the interpretation of the disappearance curve of 4-C^{14}-cortisol, undoubtedly aldosterone, both in the initial rapid and later slower phase of movement, distributes itself into much larger volumes than cortisol. Corticosterone behaves in a manner similar to cortisol in this regard (2). As cortisol and corticosterone are strongly bound to a specific plasma protein ("transcortin") (15–17) and aldosterone seems to be much more weakly bound (18), this may be one explanation for the difference in the distributions of the steroids. An analogous situation has been noted for the transport of triiodothyronine and thyroxine (19).

The slope (β) of the latter part of the curve for the disappearance of 7-H^3-aldosterone is about 30 ($T_{\frac{1}{2}} = 33$ minutes) and this is much greater than for cortisol (β value 12.8, $T_{\frac{1}{2}} = 78$ minutes). This indicates that the rate of metabolism of aldosterone is greater than that of cortisol. But the value does not represent metabolism alone. A more valid method of comparison of the metabo-

lism of steroids would be similar to that adopted by Pearlman (20). Thus, secretion rate = M × plasma concentration, where M can be defined as the turnover rate of the plasma and $M = V_1 \times K_2$. This turnover rate (M) seems a reasonable measure of the over-all metabolism of a steroid for the purposes of comparison.

The M value is dependent upon whether a single- or double-compartment model is used for the calculation. If M_1 is the value for the single-compartment model and M_2 is the value for the two-compartment model, then

$$M_1 = \beta \times \bar{V} = \frac{\beta}{B};$$

$$M_2 = V_1K_2 = \frac{\alpha\beta(A + B)}{A\beta + B\alpha} \times \frac{1}{A + B}$$

$$M_2 = \frac{\alpha\beta}{A\beta + B\alpha} = \frac{\beta}{B} \times \frac{1}{1 + \frac{A}{B} \times \frac{\beta}{\alpha}}$$

$$M_2 = M_1 \times \frac{1}{1 + \frac{A}{B} \times \frac{\beta}{\alpha}}.$$

For cortisol the values of M_1 and M_2, calculated from the data of Peterson (2), are 200 and 173 L per day, respectively. M_1 and M_2 values for aldosterone differ by a much larger factor. Using the mean value for normal subjects (Table I), M_2 = 1,620 and M_1 = 2,370. It is therefore necessary to use the M_2 value for calculations of the turnover rate of aldosterone.

However, whatever model is used, there is little doubt that the turnover rate for aldosterone is much greater than that of cortisol. The turnover rates of cortisol and corticosterone are similar (2). As pointed out by Bartter and Mills (21), the difference in the turnover rates of the three hormones could be related to the lower binding of aldosterone to plasma proteins compared with that of the other two hormones. This may result in a greater speed of movement of aldosterone from the plasma into the metabolic cells of the liver. In addition, aldosterone is metabolized to the "3-oxo conjugate" which may be more rapidly formed than the usual tetrahydro metabolites of cortisol.

A knowledge of the turnover rate can be used to calculate the mean plasma concentration from the secretion rate of the hormone, obtained from the specific activity of a urinary metabolite. Table II shows the values for the secretion rates, turnover rates and the calculated mean daily plasma concentration for the five subjects investigated. The average plasma concentration was 0.0077 (0.0026 to 0.015) μg per 100 ml plasma. The values for M_2 are fairly constant compared with the variation in secretion rates. In previous studies of 11 normal women the average secretion rates have been estimated as 140 (50 to 235) μg per day (3, 22). Using the mean value for M_2 obtained in the present studies, the mean plasma concentration can be estimated as 0.009 μg per 100 ml plasma. The plasma concentrations by direct methods (1) have given higher values; but these samples were all drawn in the morning and it is now known that there is a diurnal variation in the excretion of aldosterone (23) for subjects engaged in normal activity. Hence, 0.01 μg per 100 ml plasma may be a reasonable mean daily concentration. In a preliminary study on one patient (1) an assumption was made that the volume of distribution of aldosterone was of the same order as that of cortisol. The M_1 value was calculated to be 800. The present studies using two compartments show this to be in error. The difference between the calculated M values of 800 and the present 1,620 emphasize the importance of calculating the volumes of distribution in more than one compartment.

TABLE II

Calculated values for turnover rates (M_2), secretion rates and mean plasma concentration in five normal women

Subject	M_2	Secretion rate	Mean plasma conc.
	L./day	*μg./day*	*μg./100 ml plasma*
Patient E.A.	1,710	255	0.0149
Patient M.C.	1,370	59	0.0043
Patient A.A.	1,480	103	0.0069
J.D., normal	1,730	45	0.0026
A.M., normal	1,810	179	0.0099
Mean value	1,620	128	0.0077

The values of the turnover rate are not dependent (to within 40 per cent error) on whether a single or more complex model is used to interpret the results. But the penetration of steroids into various tissues, which is important in considering their mode of action, is critically dependent upon which model is adopted. Thus, the vol-

ume of distribution of aldosterone according to the single-compartment model is about 80 L ($\bar{V}$) for some subjects (Table I). As this is greater than the total body water volume, the data suggest that a considerable amount of steroid is held in tissues at a concentration greater than in the plasma. However, the appropriate volume given by the two-compartment model is 40 L ($V_1 + V_2$). This smaller value does not require this explanation. Similar considerations hold for calculated volumes of distribution of cortisol, where $\bar{V}$ is 16 but $V_1 + V_2$ is 13 L. The dangers of regarding the final slope of the curve as being entirely due to metabolism are also illustrated in this study, as the calculated K_2 value for aldosterone is about one-half the final slope β.

However, although the two-compartment model has the advantage of giving determinate values for the volumes of distribution and also for K_1 and K_2, it is entirely possible that a more complex model is appropriate. It could be that there are additional compartments (such as the cerebrospinal fluid) into which the steroid slowly diffuses. In this case, the curve would change in slope with time even after 50 minutes. There is no evidence that this occurs from the data obtained (Figures 4 and 5). If such diffusion did occur, the calculated K_2 values and turnover rate would be too high. Also, if metabolism occurred in the outer pool, the turnover rate calculated from the single-injection data would again be inaccurate, and the β value would more truly reflect the over-all metabolic processes.

The validity of the calculations for a two-compartment system have been tested independently by the results observed from a constant infusion experiment. After the initial priming dose, steroid has been continuously infused. The radioactivity in the plasma measured specifically as the steroid remained constant.

Considering $i \equiv$ the final constant radioactivity of the steroid per liter of plasma and $C \equiv$ rate of infusion of radioactivity as steroid per day, then $C/i \equiv$ turnover rate M. During the constant infusion of the radioactive steroid for 90 minutes after a priming dose, the radioactivity was found to be constant within the limits of experimental error (Figure 6). The M value calculated from the final plasma radioactive concentrations was found to be 1,700 L per day which agrees with the M_2 value (mean 1,620 L per day) calculated from the single-injection data using the two-compartment model as previously specified.

It therefore appears that the value of the turnover rate calculated from the data obtained after a single injection of radioactive hormone

$$\left(M_2 = V_1K_2 = \frac{\alpha\beta}{A\beta + B\alpha}\right)$$

is valid. It should now be possible by using the methods described here to compare the mean concentrations and over-all rates of metabolism of aldosterone in various clinical conditions.

SUMMARY

Studies are described on the disappearance of the radioactivity in plasma after the injection of 0.1 μg, 2 μc of 7-H³-*d*-aldosterone into five normal woman. The radioactivity was measured specifically as aldosterone and followed for 70 minutes in three subjects and for 180 minutes in two subjects.

The disappearance curve was similar in all normal subjects studied. There was an extremely rapid initial drop in plasma radioactive concentration to less than 5 per cent of the dose per L of plasma immediately after injection. The curve then became less steep, with a half-life of about 14 minutes, until about 30 minutes after injection. At this time, the plasma concentration was about 1 per cent of the dose per L of plasma. The curve then flattened again and became a straight line (semilog plot) at 30 minutes for most subjects. The half-life of this slope was about 35 minutes. From these data it can be concluded that aldosterone spreads into a much greater volume and its rate of metabolism is higher than that of cortisol and corticosterone.

As a result of mathematical analysis in terms of a two-compartment model, the data have been interpreted to mean that aldosterone spreads rapidly into a volume of 25 L and then more slowly to 40 L. The initial volume has a metabolic half-life of about 15 minutes.

The mean turnover rate of aldosterone in unit volume of plasma, 1,620 L per day, has been calculated from these data. Combining this value with the concomitant mean secretion rate estimates

(urinary metabolite method) of 128 μg per day, the mean plasma concentration of aldosterone for the five normal subjects has been calculated to be 0.0077 μg per 100 ml of plasma.

The turnover rate has been confirmed by a constant infusion experiment on one woman.

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THE ASSAY AND NATURE OF FOLIC ACID ACTIVITY IN HUMAN SERUM *

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It has been generally accepted that the folic acid nutritional status of man could not be assessed by whole blood or serum sampling (1–3). Recently, however, the subject was reopened with the finding that serum folic acid activity, as measured by *Lactobacillus casei* (ATCC [1] no. 7469), is apparently an accurate measure of the nutritional status of man with respect to folic acid (4–7).

The present report indicates that the folic acid activity in human serum may be mainly due to one or more reduced triglutamyl (or higher) forms of folic acid. Some of the implications of this probability will be considered.

Incidental to these studies, the original assay method was further modified, and an explanation for the failure of prior studies by others was found in the lability of the serum folic acid activity measured. A preliminary report of these studies has been presented (8).

MATERIALS AND METHODS

Our standard method for assay of serum folic acid activity was as follows.

Preparation of serum samples. Blood was obtained from fasting subjects using either acid-washed sterile syringes or Vacutainers no. 3200 (Becton, Dickinson & Co., Rutherford, N. J.) to ensure freedom from contamination with traces of folic acid. The blood was allowed to stand for approximately 3 hours at room temperature (in the original Vacutainer or after transfer of blood from syringes to acid-washed screw-top tubes). The clots were "rimmed" with glass rods or wooden applicator sticks, the tubes centrifuged for 5 minutes at 3,000 rpm, and the supernatant serum aspirated and frozen at − 20° C until assay. On the day of assay, the sera were thawed by standing at room temperature, or occasionally more rapidly by placing the tubes in warm water until almost thawed.

Serum aliquots were prepared for assay by diluting 1 : 10 with 0.05 M sodium phosphate buffer at pH 6.1 (9) in which ascorbic acid, in the concentration under study, had been freshly dissolved. The serum-buffer solution was autoclaved for 10 minutes at 118° C, without preliminary incubation. In the earlier method (5), preliminary incubation at 37° C for 90 minutes was part of the protocol; this has proven superfluous (10, 11). The white protein coagulum was then thrown down by centrifugation for 5 minutes at approximately 3,000 rpm and the clear supernatant assayed. This method of preparation of serum samples is similar to the method of preparation of whole blood by Toennies, Usdin and Phillips (12).

Maintenance of assay organisms and preparation of inocula. L. casei, Streptococcus faecalis (ATCC no. 8043), and *Leuconostoc citrovorum* (*Pediococcus cerevisiae,* ATCC no. 8081) were maintained in a medium previously described for maintenance of *L. casei* (5), and stored at 4° C. This medium proved excellent for maintaining all three microorganisms.

De-ionized water [2] was used for preparation of all media and buffers. It was free of folic acid and more easily prepared than distilled water. The heavy metal ions in distilled water have the undesirable ability to accelerate oxidation of the ascorbic acid in the buffer.

Five hundred ml of maintenance medium was generally prepared at one time and dispensed into 50 screw-capped tubes. The tubes, with screw caps loosely affixed, were autoclaved for 30 minutes at 118° C. They were then allowed to cool, the screw caps were tightened and the tubes incubated overnight at 37° C. The following morning they were examined for clarity (indicating absence of bacterial contamination) and, if sterile, were then stored at 4° C until used. At a maximal interval of once every 2 weeks, 1 drop of each stored liquid culture was added to 10 ml of fresh maintenance medium, incubated 18 hours at 37° C, and then stored at 4° C. During the afternoon of the day prior to an assay, 1 drop of the latest stored culture was added to 10 ml of maintenance medium, and incubated for 18 hours at 37° C. The next

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[1] American Type Culture Collection, Washington, D. C.

[2] Tap water passed through a Barnstead Bantam standard demineralizer cartridge no. 0802 (Barnstead Still and Sterilizer Co., Boston, Mass.).

TABLE I

Assay protocol finally adopted as the "standard method" of determination of folic acid activity in serum

Flask		Double-strength basal medium	Ion-free water	Buffer*	Folic acid standard	Serum diluted in buffer*
Number	Nature					
		ml	*ml*	*ml*	*ml*	*ml*
1, 8	Control	5	4	1	0	0
2, 9	Standard	5	3	1	1 (10^{-10} g/ml)	0
3, 10	Standard	5	1	1	3 (10^{-10} g/ml)	0
4, 11	Standard	5	3	1	1 (10^{-9} g/ml)	0
5, 12	Standard	5	1	1	3 (10^{-9} g/ml)	0
6, 13	Standard	5	3	1	1 (10^{-8} g/ml)	0
7, 14	Standard	5	1	1	3 (10^{-8} g/ml)	0
15, 16	Unknown serum 1	5	4	0	0	1
17, 18	Unknown serum 2	5	4	0	0	1

* Sodium phosphate buffer, 0.05 M, pH 6.1, containing 150 mg per cent ascorbic acid.

morning, 0.5 ml of this fresh 18-hour culture was added to 10 ml of maintenance medium and incubated for 6 hours at 37° C. The inoculum was prepared by adding 0.5 ml of this fresh 6-hour culture to 10 ml of single-strength basal medium. One drop of inoculum was added to each flask.

Basal media. The assay medium for *L. casei* has been previously described (5). This medium was almost identical with the basal medium used by Jukes (13) for *L. casei*, differing in only two respects, neither crucial. It should be noted that the *L. casei* basal medium of Jukes was only slightly modified from the medium for *S. faecalis* of the Association of Official Agricultural Chemists (14), which in turn derived from that of Teply and Elvehjem (15). In our basal medium: *1*) a commercial salt-free hydrochloric acid hydrolysate of casein (Hy-Case, salt-free; Sheffield Chemicals, Norwich, N. Y.) was used instead of preparing it in the laboratory. An almost identical simplification of *L. casei* assay media preparation had been used previously by others (12) who used a similar, but not salt-free, hydrolysate. *2*) The amount of para-aminobenzoic acid used was twice that used by Jukes. This was done to counteract any inhibitory effect on growth should any of the sera assayed contain appreciable amounts of sulfonamides. The reader is referred to prior publications for a tabular listing of the ingredients of the medium (5, 16) and for the method of preparing each ingredient prior to use (13, 14, 16). See the Appendix for a detailed summary.

Different commercial basal media for *S. faecalis* and *L. citrovorum* (17) were used in initial studies, but were discarded when it was found that our *L. casei* basal medium allowed as good or better growth of these two organisms as did the commercial media specially formulated for them.

Volatile preservative (VP) was added to all stored solutions of assay medium and of buffer by spraying a small aliquot from a wash bottle into each solution after each use. VP consisted of one part ethylene dichloride, one part monochlorobenzene, and two parts 1-chlorobutane (18).

Standards. Three standard solutions of folic acid (pteroylglutamic acid, PGA) were prepared: 10^{-8}, 10^{-9}, and 10^{-10} g per ml. Similar standard solutions of folinic acid (N^5-formyl-tetrahydrofolic acid, citrovorum factor) were prepared by diluting calcium leucovorin [3] to concentrations twice those of the folic acid standards. Since calcium leucovorin is a synthetic racemic form of folinic acid (citrovorum factor) (19), such dilution resulted in concentrations of folinic acid activity equivalent to the folic acid activity of the standard solutions of folic acid. Weight for weight, we found calcium leucovorin to be approximately half as active for *L. casei* as folic acid. Dawbarn, Hine and Smith (20) had found calcium leucovorin to be 38.3 per cent as active for *L. casei* as folic acid. It is possible that in our assay system, some of the inactive *d*L-isomer may be converted to the active *l*L-isomer. This has been reported to occur in the presence of dilute acid (21), and *L. casei* gradually makes its assay medium more acid as it grows and produces lactic acid.

Assay protocol. All glassware was rendered folic acid-free by rinsing thrice in tap water, boiling gently in Haemo-sol (Meinecke & Co., New York, N. Y.) solution for 0.5 hour, rinsing 12 times in tap water, thrice in de-ionized water, and then drying in air. The aluminum caps which covered the flasks were rendered folic acid-free by rinsing 12 times in tap water, then thrice in de-ionized water. They were not washed in detergent because it proved unnecessary (and it corroded the metal).

With an automatic pipet, 5 ml of double-strength basal medium was added to each 10 ml micro-Fernbach flask (18) (10 ml Erlenmeyer flasks are less expensive, equally good, but more susceptible to tipping over). From 1 to 4 ml of de-ionized water was next added to each flask, the volume being such as to bring the total volume in each flask to 10 ml when all ingredients had been added.

Table I illustrates the assay protocol of our standard method for the determination of folic acid activity in serum. After all ingredients had been added, the flasks

[3] Kindly provided by Drs. T. H. Jukes and E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y.

were autoclaved for 30 minutes at 105 to 110° C, allowed to cool, and inoculated.

One drop of inoculum was added to each flask, and the flasks incubated [4] 16 to 18 hours at 37° C. Each flask was then vigorously shaken and its content transferred to a Klett colorimeter tube. Growth density was then measured with a Klett-Summerson photoelectric colorimeter [5] using a red filter ($\lambda = 640$ to 700 mμ) to reduce error due to the color of the medium. The growth densities of the standards were plotted on semilogarithmic paper and serum values calculated from the curve (5). Usually the standards were run in duplicate, the results averaged, and the average values plotted. Duplicates rarely differed significantly.

Aseptic addition method. In experiments using unheated serum, 0.1 ml aliquots of serum were added aseptically to autoclaved solutions containing 5 ml of double-strength medium, 4 ml of de-ionized water, and 1 ml of stated concentrations of ascorbic acid in phosphate buffer. This is referred to below as the "aseptic addition method."

All experiments involving more than 350 mg per cent ascorbic acid [6] were done by the "aseptic addition method," because such amounts of ascorbic acid prevented serum protein from being thrown down in the centrifugation of the standard method. The dense heated protein markedly raised the background turbidity, masking that due to bacterial growth.

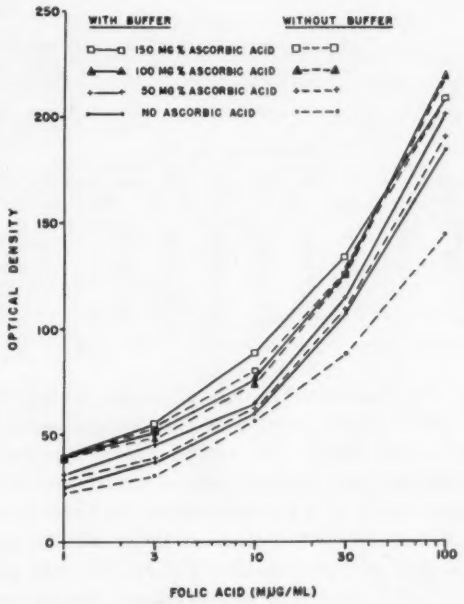

FIG. 1. ENHANCEMENT OF *L. casei* GROWTH BY ASCORBIC ACID AND PHOSPHATE BUFFER.

RESULTS

Effect of ascorbic acid, phosphate buffer, and heat

1. Effect on growth of L. casei standards. Employing our standard method, *L. casei* standard curves were obtained, adding 1 ml of varying concentrations of ascorbic acid with and without phosphate buffer. Figure 1 demonstrates that the growth of *L. casei* is stimulated by both ascorbic acid and phosphate buffer. Stimulation by ascorbic acid increased with increasing amounts of this agent up to 150 mg per cent. The addition of 1 ml of ascorbic acid solution in concentrations above 150 mg per cent and up to 1,500 mg per cent had no further marked stimulatory effect, and amounts in excess of 1,500 mg per cent *inhibited L. casei* growth (Table II). Adding the standard solutions of folic acid (PGA) to the assay medium after autoclaving did not result in any increase of *L. casei* growth over that obtained when folic acid was added before autoclaving (data not shown).

2. Effect on ability of human serum to stimulate growth of L. casei. Sera were prepared for assay by four different methods: Method 1, our standard method; Method 2, 1 ml serum was diluted to 50 ml with distilled water, and 5 ml of this diluted serum was added to 5 ml of double-strength medium which was then autoclaved and inoculated (this was the method used by Chanarin, Anderson and Mollin (*22*) for preparing serum for "folic acid" assay with *S. faecalis*); Method 3 same as Method 2, but with 9 ml of 150 mg per cent ascorbic buffer replacing 9 ml of the water used to dilute the serum; Method 4, the aseptic addition method, using 150 mg per cent ascorbic buffer. Since Methods 2 and 3 produced a serum protein precipitate in the incubation medium, non-inoculated control sera were run, and the optical density of the controls was subtracted from the optical density reading of the inoculated samples. To

[4] American standard incubator, A. H. Thomas Co., Philadelphia, Pa.

[5] Klett Manufacturing Co., New York, N. Y. The optical density units employed in the present paper are the scale readings of this instrument. The scale readings are directly proportional to concentration when Beer's law holds.

[6] "Mg per cent ascorbic acid" will always refer to mg per cent ascorbic acid in the 1 ml of buffer of column 5 or column 7 in Table I. Thus, *the actual concentration of ascorbic acid in 10 ml of finished medium is one-tenth the amount stated.*

TABLE II

Effect of increasing concentrations of ascorbic acid from 100 to 15,000 mg per cent on growth of L. casei in the presence of varying amounts of folic acid

	Growth of *L. casei* (in optical density units) in medium containing varying amounts of added ascorbic acid in 1 ml of phosphate buffer														
	Ascorbic acid (mg %)						Ascorbic acid (mg %)						Ascorbic acid (mg %)		
Folic acid	Expt. A	100	150	200	250	350	Expt. B	150	500	1,000	1,500	2,000	2,500	Expt. C	150	1,500	15,000
mμg/ml				*mu*							*mu*					*mu*	
0		44	49	47	48	50		38	39	31	33	32	29		37	39	8
0.1		49	56	50	53	51		47	46	43	47	43	36		48	49	7
0.3		50	61	61	61	67		67	67	64	67	62	52		62	67	8
1		65	80	80	81	84		111	115	115	116	103	90		94	95	8
3		98	110	113	105	109		163	165	160	160	155	133		140	138	9
10		163	165	172	170	165		228	228	226	230	213	210		204	205	9
30		221	195	211	209	196		250	248	252	245	255	235		226	222	9

this value was added the optical density reading of an uninoculated control flask containing no added folic acid or serum. The resulting figure was used to calculate folic acid activity. Chanarin and associates (*22*) do not record adding the reading of an inoculated control flask in their calculations. This may explain why they find *no* folic acid activity for *S. faecalis* in fasting serum, whereas we consistently find a small amount of such activity using their methodology, but compensating for growth occurring in the absence of added serum.

The "folic acid activity" of the sera was calculated against the appropriate standard curves (i.e., Methods 1, 3, and 4 were calculated against a standard with added ascorbic buffer, and Method 2 was calculated against a standard without added ascorbic buffer).

Table III shows results obtained with sera from patients with poor, marginal, and excellent folic acid nutritional status.[7] In the presence of 1 ml of 150 mg per cent ascorbic buffer, regardless of whether or not the serum was heated (Methods 1, 3 and 4), the serum folic acid activity for *L. casei* reflected the patient's nutritional status with respect to folic acid. On the other hand, when the sera were heated in the absence of ascorbic acid (Method 2), the folic acid activity for *L. casei* of sera from the patients with excellent and marginal folic acid nutrition fell as low as that of the patient with poor folic acid nutrition.

Incubation of serum from normal subjects in the absence of added ascorbic acid at 37° C for 18 hours also resulted in loss of all activity for *L. casei*, as measured by our standard method (data not shown).

Both the phosphate buffer and ascorbic acid protect serum folic acid activity for *L. casei* even when the serum is not heated ("aseptic addition method," Table IV; Figure 1 depicts the standard curves for Experiment A of Table IV).

Protection by ascorbic acid increases as its concentration rises to 150 mg per cent. Increasing the amount of ascorbic acid from 150 to 350 mg per cent slightly increased the protective effect over that obtained with 150 mg per cent ascorbic acid (Table IV, Experiment B; the standard curve for this experiment is recorded in Table II, Experiment B).

The serum folic acid activity is unaffected by raising the amount of added ascorbic acid from 1 ml of 500 mg per cent to 1 ml of 1,500 mg per cent (Table IV, Experiment C). There is an *ap-*

[7] Basis of classification: Poor = diet devoid of meat, fruit, vegetables, normal serum vitamin B_{12} level; megaloblastic anemia responsive to folic acid. Marginal = diet poor in sources of folic acid; normal serum vitamin B_{12} level; slight macrocytosis and increase in segmentation of nuclei of polymorphonuclear leukocytes without overt megaloblastic anemia. Excellent = good diet; no signs or symptoms of malabsorption; normal serum vitamin B_{12} level; no macrocytosis or increase in segmentation of nuclei of polymorphonuclear leukocytes.

TABLE III

Effects of heat and ascorbic acid on serum folic acid activity

Serum treatment			Folic acid nutritional status of serum donor		
Heat	Ascorbic acid	Method*	Poor	Marginal	Excellent
			Serum folic acid activity		
			mμg/ml		
Yes	Yes	1	2.1	6.3	29.0
Yes	No	2	2.3	1.8	2.9
Yes	Yes	3	3.3	4.1	28.0
No	Yes	4	2.1	4.0	24.0

* See text.

parent rise when the added ascorbic acid is raised to 1 ml of 2,500 mg per cent, but this is due to inhibition of the standard curve growth by this amount of ascorbic acid (indicated in Table II, Experiment B); this rise therefore is apparent but not real. It may be due to the presence in serum of various ascorbic acid-inactivating substances, which would reduce the actual ascorbic acid level in the sera below the toxic level in the standards. The inhibition of growth by large amounts of ascorbic acid was probably not due to lowering of pH, since in the experiments recorded in Tables II and III the ascorbic acid solutions were all titrated to pH 6.5 with 10 per cent KOH before they were used. However, "moderate changes in the buffering capacity of the medium, such as may result from the buffering power of the added analytical specimen, may significantly affect growth response" (23).

Substitution of d*D-iso-ascorbic acid or 2-mercaptoethanol for ascorbic acid.* One hundred fifty mg per cent D-iso-ascorbic acid (D-araboascorbic acid) (D-erythro-3-ketohexonic acid lactone) is an effective substitute for the same amount of ascorbic acid (or sodium ascorbate) in the buffer used to prepare sera for assay, as indicated by the fact that sera prepared with either agent assayed almost identically (Table V, Experiment D). However, D-iso-ascorbic acid provided less stimulus than ascorbic acid to growth of the *L. casei* standard curves, and was therefore not further studied as a possible replacement for ascorbic acid, because for assay purposes it was desired to provide, insofar as possible, an excess of every *L. casei* active material that human serum contains *except folic acid active materials.* Human serum ordinarily contains ascorbic acid.

TABLE IV

Protective effect of phosphate buffer and ascorbic acid

		Ascorbic acid	Serum folic acid activity	
			Serum diluent	
			Water	Buffer
		mg %	*mμg/ml*	
Expt. A	Serum 1	0	6	8
Aseptic		50	5	8
addition		100	7	10
method		150	14	15
Expt. B	Serum 2	100		7
Standard		150		13
method		200		15
		250		17
		350		17
Expt. C	Serum 3	500		8
Aseptic		1,000		8
addition		1,500		8
method		2,000		10
		2,500		14

Using the "aseptic addition method," it was found that 1 ml of 2 g per cent 2-mercaptoethanol substituted completely for ascorbic acid in protecting serum folic acid activity, whereas 1 ml of 0.2 g per cent 2-mercaptoethanol afforded only slight protection (data not shown).

Using the "standard method," 0.2 g per cent 2-mercaptoethanol protected serum folic acid activity (Table V, Experiment E). This concentration of 2-mercaptoethanol prevented protein precipitation, making necessary the use of a control (not inoculated) flask.

Unlike ascorbic acid (and D-iso-ascorbic acid, to a lesser extent), 2-mercaptoethanol did not stimulate *L. casei* growth. Addition of 1 ml of 2

TABLE V

Protection of serum folic acid activity on heating in the presence of 150 mg per cent ascorbic acid or D*-iso-ascorbic acid, or 0.2 g per cent 2-mercaptoethanol*

	Serum	150 mg % ascorbic acid	150 mg % D-*iso*-ascorbic acid	200 mg % 2-mercaptoethanol
		Serum folic acid activity		
	no.	*mμg/ml*	*mμg/ml*	*mμg/ml*
Expt. D	4	8.0	7.0	
	5	11.3	9.4	
	6	2.8	3.4	
	7	4.1	4.7	
Expt. E	8	12.5		13.4
	9	1.3		<1.0

TABLE VI

Similarity of the folic acid activity of serum and plasma

Subject	Serum Folic acid activity	Plasma
	mμg/ml	
10	3.7	3.6
11	4.4	4.0
12	6.9	5.3
13	10.3	12.5
14	15.8	14.3

g per cent 2-mercaptoethanol to the medium slightly inhibited *L. casei* growth in the presence of 1 ml or more of 10^{-8} g per cent PGA but not with lesser PGA concentrations (data not shown).

Folic acid activity of serum versus plasma. Table VI indicates that the folic acid activity of serum prepared as indicated in Materials and Methods is essentially the same as that of plasma obtained from the same subjects at the same time. The plasma was obtained by centrifugation of whole blood *immediately after withdrawal* from subjects into Vacutainers, no. 3204, containing dry balanced oxalate with immediate aspiration and freezing until assay of the plasma layer after centrifugation.

Activity of serum for L. casei versus S. faecalis versus L. citrovorum. Table VII shows that 1 ml of serum from normal subjects markedly stimulates microbiological growth (exceeding that produced by 5 mμg of folic acid) only when supplied to *L. casei*. The growth of *S. faecalis* and *L. citrovorum* is slight on all human sera studied so far, and neither of these microorganisms grows substantially better on sera from normal human subjects than on sera from folic acid-deficient subjects. This is true whether the sera are prepared for assay by autoclaving in the phosphate-ascorbic acid buffer or by aseptic addition to pre-autoclaved ascorbic acid-containing media (Table VII).

TABLE VII

Folic acid activity of sera determined by standard method or aseptic addition method with L. casei, S. faecalis and L. citrovorum

	Serum	*L. casei* Serum folic acid activity	*S. faecalis*	*L. citrovorum*
		mμg/ml		
Expt. F	15	1.6	1.6	1.6
Aseptic	16	2.7	4.1	2.5
addition	17	6.5	1.8	1.7
method	18	7.7	2.2	1.6
	19	12.2	1.9	1.6
Expt. G	20	2.9	1.1	1.0
Standard	21	6.5	1.0	<1
method	22	8.6	1.5	<1
	23	11.3	1.1	<1
	24	34.0	1.7	<1

DISCUSSION

L. casei growth is stimulated by both ascorbic acid and phosphate buffer. Therefore, serum "folic acid" values obtained by using ascorbic acid-phosphate buffer in the sera and not in the standards would give erroneously high results. In our prior studies of serum "folic acid" activity (4–6) this error was made. Fortuitously, the results obtained by that method compare fairly well with results obtained by the method here reported, because the lesser growth of *L. casei* in the standards in our prior studies (due to the absence of ascorbic acid) was counterbalanced by lesser growth in the sera, due to incomplete protection of serum folic acid activity by 50 mg per cent ascorbic buffer. The fact that ascorbic acid per se enhances growth of *L. casei* was previously observed by other workers (24, 25). This important consideration was not taken into account in our prior studies (4–6), or in most of the reported studies on the folic acid content of foods. For this reason, a significant amount of the reported "folic acid" content of diets in various studies may in fact be ascorbic acid content.

The studies here reported indicate the great lability of serum folic acid activity if it is not adequately protected by ascorbic acid. This protection appears to be afforded by the reducing ability of ascorbic acid. This is indicated by the fact that D-iso-ascorbic acid, which is biologically relatively inactive except as a reducing agent (26), affords protection similar to that provided by ascorbic acid. 2-Mercaptoethanol, a less potent reducing agent, also provides protection when it is in adequate concentration.

The lability of the serum folic acid activity was first suspected from initial studies in Boston, where we consistently obtained lower values for serum folic acid activity than we did in New York where a large steam autoclave with reliable temperature control was used. The difficulty was traced to the

use in Boston of a small electric autoclave that failed to produce a rapid rise to pre-set temperature and maintained poor temperature control thereafter. This resulted in a more prolonged exposure part of the time to higher temperatures. Recognition of the source of the difficulty led to the present use of 150 rather than 50 mg per cent ascorbic acid, which protects the serum folic acid activity against thermal vicissitudes. It is possible that a similar problem may have beset other workers (*27*) who were unsuccessful in an attempt to reproduce our results, using modification of our method. They autoclaved sera at 15 psi, which is 121° C when the autoclave is functioning properly and there is complete replacement of air by steam (*17*). At that temperature, serum folic acid activity is destroyed in only 50 mg per cent ascorbic acid. Their use of that pressure may have derived in part from the error in our paper (*5*), stating that autoclaving was performed at "118° C, 16 psi." The 118° C was correct; 16 psi was incorrect. At 16 psi, assuming a properly functioning autoclave and complete replacement of air by steam, the temperature would be 122° C (*17*). The finding by Cooperman, Luhby and Avery (*27*) of marked folic acid activity in sera from folic acid-deficient subjects may have been due to contamination of serum samples with folic acid activity. A major source of such contamination in our early studies was the use of non-acid-washed syringes.

The three changes from our original (*5*) protocol and the reasons therefore are: *1*) 1 ml of phosphate-ascorbic acid buffer is added to each standard because the buffer itself stimulates *L. casei; 2*) for the same reason 1 ml of serum diluted in buffer is used instead of the 0.5, 1, and 1.5 ml amounts previously used, because varying amounts of phosphate-ascorbic acid buffer provided varying stimulation of *L. casei; 3*) 150 mg per cent ascorbic acid is used because 50 mg per cent is not adequate to protect serum "folic acid" from accidentally higher than normal autoclave temperatures.

Toennies and co-workers (*12*) found that ascorbic acid in excess of 50 mg per cent interfered with protein coagulation of whole blood diluted 750-fold. Fortunately for our "standard method," amounts of ascorbic acid up to 350 mg per cent do not interfere with protein coagulation of serum diluted 10-fold.

During the course of a report (*12*) on precursors of the folic acid-active factors in blood, it was stated that normal plasma is nearly inactive for *L. casei*. Because of that statement, it seemed possible that what we refer to as "*serum* folic acid activity" was in fact derived from the large quantity of folic acid-active material in erythrocytes (*12, 28, 29*) during the 3 hour period the clotted blood stands at room temperature prior to removal of the serum. The present finding that *serum* folic acid activity and *plasma* folic acid activity are approximately the same indicates that this activity is not derived from the cellular components of blood *in vitro*, since the plasma was removed from whole blood immediately after venipuncture.

After ingestion of 15 mg of folic acid, a "labile citrovorum factor" appears in the urine (*30*). Like "serum folic acid activity," this material is inactivated by autoclaving in the absence of ascorbic acid, but is protected (presumably against oxidative destruction) by the presence of ascorbic acid. The material with these characteristics appears to be a derivative of N^{10}-formyltetrahydrofolic acid (*30*).

Serum folic acid activity differs from the "labile citrovorum factor" in urine in at least one important respect: it has very little growth activity for *L. citrovorum*. The evidence that serum folic acid activity may be due mainly to a derivative of N^{10}-formyltetrahydrofolic acid, and the seemingly opposed fact that despite autoclaving in the presence of ascorbic acid, which should have converted it to folinic acid (*30*), it has little growth activity for *L. citrovorum*, requires explanation. Serum protein binding appears to play no role in the relative unavailability of serum folic acid activity for *S. faecalis* and *L. citrovorum*, because these organisms do not grow markedly on human serum whether or not serum protein is precipitated by autoclaving prior to assay.

The most obvious explanation for *L. casei* activity at a higher level than *S. faecalis* or *L. citrovorum* activity is the presence of folic acid-active materials at the triglutamyl (or higher) level (*29, 31, 32*). The latter two organisms grow slightly or not at all on such materials. The folic acid-active diglutamate, pteroyl-γ-glutamyl-glutamic

acid, is active for both *L. casei* and *S. faecalis;* the diglutamate N^5-formyltetrahydrofolylglutamate is active for all three microorganisms; all the known folic acid-active monoglutamates are active for *L. casei* and for either or both *S. faecalis* and *L. citrovorum* (29, 31). The latter organism is unique in its requirement for a reduced form of folic acid (32). Folic acid-active polyglutamates isolated and characterized from natural materials appear to have three or seven glutamic acid residues (32). Since *L. casei* does not grow appreciably on heptaglutamates (31–33) it is reasonable to infer that serum folic acid activity is due mainly to *tri*glutamates.

It was previously suggested (5) that utilization of PGA polyglutamates may be the crucial property contributing to the superiority of *L. casei* over *S. faecalis* for the assay of serum folic acid activity. This superiority was assumed by comparison of our *L. casei* results with the *S. faecalis* results of Chanarin and co-workers (22). However, in light of the present studies of the lability of serum folic acid activity, the failure to find folic acid activity in fasting serum using *S. faecalis* may have been due to the failure to protect serum folic acid activity with ascorbic acid. The present study demonstrates that even with such protection serum folic acid activity is unavailable to *S. faecalis*. The present study therefore indicates that serum folic acid activity is due mainly to polyglutamates, but suggests that they are polyglutamates of reduced forms of PGA, rather than of PGA itself.

In cell-free bacterial enzyme systems, tetrahydrofolic acid (THFA, which has one glutamic acid residue) appears catalytically inactive as a one-carbon unit carrier; this pteridine coenzyme becomes catalytically active only when it has three glutamic acid residues—i.e., when it is a polyglutamate (34). This catalytic activity is dependent upon the presence of DPN, Mn^{++}, and pyridoxal phosphate (34). It is possible that in all bacterial enzyme systems a polyglutamyl derivative(s) is the true cofactor form of folic acid and that in the crude systems, when the monoglutamyl derivatives are active, a mechanism is available for the synthesis of the derivatives with more than one glutamic acid present (35).

In studies with partially purified cell-free systems of mammalian (rabbit) liver, THFA appears to be catalytically active as a one-carbon unit carrier in the presence of pyridoxal phosphate (36). The experimental data suggested, however, that conjugates of THFA possessing three or seven glutamic residues were even more active than THFA itself (36). One interpretation of these data might be that the partially purified cell-free mammalian liver preparations contained an enzymatic mechanism capable of converting THFA to a polyglutamate, and that only THFA polyglutamates are catalytically active. Conjugases for folic acid-active polyglutamates are distributed in animal tissues, especially liver and kidney (31, 37, 38). The conjugase in human blood appears able to split pteroylheptaglutamates mainly to triglutamates, and, to a lesser degree, to monoglutamates (39).

It may reasonably be inferred that an enzymatic system for synthesizing polyglutamates from monoglutamates occurs in man. Evidence favoring this possibility is the preliminary finding that when unconjugated PGA is administered parenterally to humans there is an immediate rapid rise in folic acid activity for both *S. faecalis* and *L. casei,* but within an hour the *S. faecalis* activity falls sharply while *L. casei* activity remains elevated (40).

Serum folic acid activity appears to be due mainly to triglutamyl derivatives of reduced forms of folic acid, but the precise characterization of the derivative(s) involved awaits isolation of the intact compound(s). Most of the serum folic acid activity may be due to triglutamyl derivatives of N^{10}-formyl THFA, $N^{5,10}$-methenyl THFA, and/or THFA carrying one-carbon units other than formyl. The heat instability of serum folic acid activity suggests that little or none of it is the triglutamyl derivative of folinic acid(41). Some sera may show more folic acid activity when assayed by the aseptic addition method than when assayed by the standard method (unpublished data). This suggests that a significant part of the activity of some sera may be due to the triglutamyl derivative of THFA carrying no one-carbon unit, since THFA itself assays higher by the aseptic addition method than by the standard method (42). The isolation of folic acid and citrovorum factor, rather than other THFA derivatives, from natural materials, is a reflection of the oxygen lability of THFA and its other derivatives, which are in fact probably the predominant (or only) forms in which the vitamin occurs in natural materials. By

the same token, the isolation of monoglutamates rather than polyglutamates probably reflects the instability of the latter.

Further study is needed to delineate how much of serum folic acid activity may derive from food absorbed as a polyglutamate and how much may be converted to the form or forms present in serum by enzymatic systems in intestine, liver and other tissues. The "folic acid deficiency" of patients with liver disease and idiopathic steatorrhea (7) may be related in part to inability to form folic acid-active polyglutamates due to deficiency or absence of the enzyme(s) involved in their synthesis from monoglutamates. This enzymatic system may reside not only in liver but also in intestine and other tissue.

SUMMARY

1. The previously reported serum "folic acid" assay with *L. casei* was modified by: addition of phosphate-ascorbic acid buffer to the *standards* as well as to the unknowns; use of *150* instead of 50 mg per cent ascorbic acid; use of *identical* aliquots of serum.

2. The maintenance medium and basal medium used for *L. casei* assay served equally well for *S. faecalis* and *L. citrovorum* assay.

3. The folic acid activity level of plasma is the same as that of serum.

4. Serum folic acid activity for *L. casei* appears to be due mainly to triglutamyl derivatives of reduced forms of folic acid. This is suggested by its relative unavailability to *S. faecalis* and *L. citrovorum*, and its protection during autoclaving by ascorbic acid, D-iso-ascorbic acid, or 2-mercaptoethanol. The metabolically active forms of the folic acid coenzymes in man may be reduced triglutamates rather than monoglutamates.

APPENDIX

The assay and maintenance media have previously been presented in tabular form (5), but without details of preparation. Such details are provided herewith in Tables VIII and IX. Preparation of the assay medium is tedious and fraught with possibilities for contamination with folic acid. Several investigators have been unsuccessful in preparing it. For these reasons, two firms were asked to prepare the assay medium in dehydrated form ("dry mix"). As of the present time, we have tested and found satisfactory the dry mix (lot 009-607)

TABLE VIII

Assay medium

Constituent	Amount for 4 L of double-strength medium	Concentration in final volume
		mg %
Hy-Case*	40 g	500
L-tryptophan	0.8 g	10
Adenine†‡	4 ml	0.5
Guanine HCl†‡	4 ml	0.5
Uracil†‡	4 ml	0.5
Xanthine†‡	8 ml	1.0
L-asparagine H_2O	2.4 g	30
L-cysteine HCl§	2 g	25
Riboflavin†‖¶	4 ml	0.05
Para-aminobenzoic acid†‖	8 ml	0.1
Pyridoxine HCl†‖¶	16 ml	0.2
Thiamine HCl†‖	1.6 ml	0.02
Ca pantothenate†	3.2 ml	0.04
Nicotinic acid†	3.2 ml	0.04
Biotin†¶**	1 ml	0.001
Glucose	160 g	2,000
Tween 80††	4 ml	0.005 ml
Glutathione (reduced)	20 mg	0.25
Salt mix‡‡	40 ml	0.5 ml
Na acetate (anhydrous)	160 g	2,000
K_2HPO_4	4 g	50
KH_2PO_4	4 g	50
$MnSO_4H_2O$§§	0.8 g	10

* Salt-free hydrochloric acid hydrolysate of casein, Sheffield Chemicals, Norwich, N. Y. Dissolve in 2 L of de-ionized water with gentle heating and stirring.

† California Foundation for Biochemical Research, Los Angeles, Calif.

‡ Solution (10 mg/ml) prepared by adding 1 g chromatographically pure reagent to 25 ml de-ionized H_2O, gently heating with added pellets of KOH until reagent dissolved, making up to final volume of 100 ml with de-ionized H_2O. Store in refrigerator with volatile preservative added.

§ Dissolve in KOH before adding.

‖ Solution (1 mg/ml) prepared by dissolving 0.1 g reagent in 100 ml de-ionized H_2O. Store in refrigerator with VP added.

¶ Store in amber polyethylene bottle (6026, Cole-Parmer Instrument and Equipment Co., Chicago, Ill.) to protect from light.

** Solution (0.08 mg/ml) prepared by dissolving 8 mg biotin in 100 ml de-ionized H_2O. Store in refrigerator with VP added.

†† Atlas Powder Co., Wilmington, Del. The viscous commercial preparation (specific gravity ≈1) is diluted 1:10 with water containing some ethanol to reduce foaming.

‡‡ One ml contains: $MgSO_4 \cdot 7H_2O$, 40 mg; NaCl, 2 mg; $MnSO_4 \cdot 4H_2O$, 2 mg; $FeSO_4 \cdot 7H_2O$, 2 mg. Make 500 ml by dissolving 20 g of $MgSO_4 \cdot 7H_2O$, 2 g of NaCl, 1 g of $MnSO_4 \cdot 4H_2O$, and 1 g of $FeSO_4 \cdot 7H_2O$ in de-ionized water, add 1 ml concentrated HCl, store with VP added.

§§ Added after pH of assay medium adjusted to 6.6 to 6.8 with 0.1 N H_2SO_4.

TABLE IX

*Maintenance medium**

Constituent	Amount for 500 ml medium	Concentration in final medium
		g/100 ml
Yeast extract (Difco)	3.75 g	0.75
Proteose peptone (Difco)	3.75 g	0.75
Glucose	5 g	1.0
KH_2PO_4	1 g	0.2
Tomato juice filtrate†	50 ml	10 ml
Tween 80‡	0.5 ml	0.01 ml
L-cysteine HCl	0.5 g	0.1

* Adjust to pH 6.8 to 7 with 1% KOH. This medium, without the L-cysteine, has been previously described (43).

† Canned or vacuum-bottled tomato juice passed through coarse filter paper; straw-colored filtrate adjusted to pH 7 with 10% KOH.

‡ See footnote††, Table VIII.

prepared by Baltimore Biological Laboratory, Baltimore, Md.

The sodium phosphate buffer (9) is prepared as follows: dissolve 27.6 g of $NaH_2PO_4 \cdot H_2O$ in 1 L distilled water (solution A); dissolve 71.6 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L distilled water (solution B). To 212.5 ml of solution A add 37.5 ml of solution B; dilute to 1 L with de-ionized water. The pH should be 6.1. Store solutions at room temperature, with volatile preservative added.

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IMMUNOLOGIC AND BIOLOGIC CHARACTERIZATION OF ANTISERA TO BEEF THYROTROPIN PREPARATIONS *

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Sensitive immunologic techniques and more purified preparations of bovine pituitary thyrotropin have made it possible to re-examine the question of whether thyrotropin acts as an antigen upon injection into heterologous species. This possibility was first indicated by Werner (1, 2) in 1936, to explain the loss of responsiveness to thyrotropin which Collip and Anderson (3) previously had observed to follow repeated injection of crude pituitary extracts. These latter workers, having demonstrated that serum from such refractory animals could neutralize the effect of thyrotropin upon the thyroid in nonrefractory animals, postulated that thyrotropic stimulation had caused an increase in the titer of an antihormone acting to preserve homeostasis by exerting an effect opposite to that of the thyrotropin. In conflict with the antihormone thesis, however, were the observations (4) that a flavianic acid preparation of thyrotropin did not evoke refractoriness, could stimulate the thyroid of the refractory animal, and could not be neutralized by the serum of refractory animals—supporting the view that antibody to a foreign protein, rather than an antihormone, had been induced. To date, however, there has been no *in vitro* demonstration of a circulating antibody specific for thyrotropin (5), nor is it known whether highly purified thyrotropic preparations evoke antibody formation.

The results of the present experiments suggest that in the rabbit, bovine thyrotropin itself, or a closely associated and as yet not separable protein, does induce the formation of precipitating antibody; and that antisera containing such antibodies can neutralize the thyrotropic effect of the hormone.

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METHODS

Thyrotropic hormone preparations. Three bovine thyroid-stimulating hormone (TSH) preparations were used in these studies. One, in clinical use under the trade name Thytropar, contained approximately 1 international unit (IU) per mg ("Armour"). The two other preparations contained greater activity ranging from 5 to 25 IU per mg, and were provided through the kindness of Dr. Peter G. Condliffe and Dr. Robert W. Bates ("Condliffe"), and Drs. John L. Pierce and M. E. Carsten ("Pierce"). Both of these latter preparations were concentrated on anion diethylaminoethyl (DEAE) and cation (carboxymethyl cellulose) exchange resins (6, 7) with some variations in methodology between laboratories. A mouse pituitary tumor thyrotropin preparation was made available by Dr. Condliffe and Dr. Bates (8) and a preparation of human thyrotropin by Dr. John Bakke and Dr. Peter Heidemann.

Pituitary hormone preparations other than TSH. A number of pituitary hormone preparations other than TSH were tested for cross reaction with the anti-TSH sera. The sources of these preparations are listed in Table II. All preparations termed "Wilhelmi" were provided by both Dr. Alfred E. Wilhelmi and Dr. Stanley Ellis. The bovine growth hormone and prolactin preparations were obtained as a gift of the Endocrine Study Section, National Institutes of Health.

Preparation of antisera. The antisera used for the precipitin and *in vivo* neutralization studies were developed in rabbits. Fifteen animals were immunized: 9 with Armour TSH, and 3 animals each with the Condliffe and Pierce TSH preparations, respectively. Three IU of thyrotropin was incorporated in 1.0 ml of Freund adjuvant and injected intracutaneously in multiple sites (9). The injections were repeated 2 or 3 times, at weekly or biweekly intervals. A total of 9 or 12 U was given to each rabbit. Ten days after the final injection, the animals were exsanguinated under sterile conditions and the sera were separated and stored without preservative at 4° C.

After the initial precipitin studies, absorption of the antisera to TSH with bovine serum was routinely performed. This was accomplished in the following manner: 0.05 ml of bovine serum was added to each 1.0 ml of antiserum and allowed to stand 5 to 7 days at 4° C. The precipitate was then removed by centrifugation, and another 0.05 ml of bovine serum added to the supernatant. This procedure was repeated until there was no further

precipitation. Absorption with hormone preparations was accomplished as with the bovine serum.

Immunological analyses. Ouchterlony plates (10) were prepared by placing 25 ml of 0.7 per cent agar in veronal or phosphate buffer, pH 7.2, in Petri plates of 9 cm diameter. Wells were so cut as to permit the selected sera and antigens to be placed in the desired relation to one another. The wells were then filled, the dishes stored at 4° C, and inspection made daily.

Immunoelectrophoretic analyses were performed according to the technique of Grabar and Williams (11). The electrophoretic separation of the antigen solutions and the subsequent precipitin reactions were carried out in a 1 per cent agar gel containing veronal buffer, pH 8.6, ionic strength of 0.025.

Bioassay for TSH activity following electrophoresis in agar. In order to establish the relationship of the immune precipitates to biologically active TSH, duplicate electrophoretic separations were performed on standard microscope slides (25 × 75 mm) coated with buffered agar (veronal, pH 8.2). The central antigen wells of these slides were filled with 0.015 ml of Armour TSH (0.15 IU per 0.015 ml) and electrophoretic separation was carried out at a potential gradient of 10 v per cm for a period of 2 hours. Following electrophoresis, a trough for antiserum was cut in the agar of one slide, parallel to the axis of electrophoretic migration, and this trough was filled with absorbed anti-TSH antiserum (C_t). Both slides were placed in a moist chamber at 4° C for 24 hours at which time a precipitation arc was visible on the slide containing antiserum. Strips of agar with a volume of 0.02 to 0.04 ml were then cut perpendicularly to the electrophoretic axis from four sites, two on the cathodal side and two on the anodal side of the antigen well at distances of 2 to 3 and 4 to 5 mm from the well. These four strips of agar were triturated in 0.5 ml of physiological saline and the suspension solution injected into mice for bioassay of TSH activity as described below. In a second experiment, the identical procedure was followed except that the agar strips were removed from the slide without antiserum immediately after electrophoresis, and the four sites were located at distances of 4 to 5 and 12 to 13 mm from the antigen well.

Bioassay methods: Tests for neutralizing activity of the anti-TSH antisera. Bioassay for thyrotropic activity was conducted by two methods. The initial assays were performed with the cooperation of Dr. Frederic Agate, Department of Anatomy. Rats were hypophysectomized and 10 days later received daily subcutaneous injections of the test preparations for a period of a week. The thyroids were then excised, weighed, and fixed in Bouin's solution. Cell height was measured according to standard technic. A minimum of 200 cells was counted per gland.

Later, assays were performed by the method of McKenzie (12), with minor modifications (13). Female Swiss mice, 15 g in weight, were fed Ken-L Bisket ration for 1 week. After this initial period, the diet was supplemented with 0.12 per cent crude thyroid, and the animals were injected intraperitoneally with 8 μc I^{131} and subcutaneously with 10 mg of sodium thyroxine. On the fifth day after I^{131} and thyroxine administration, control blood samples were obtained and the test materials were injected intravenously. Two hours after injection of the test materials, blood samples were again obtained and radioactivity measured in a well-type scintillation counter with an average background count of 192 cpm. Groups of 4 mice were used for each test point.

Testing of the antisera for antithyrotropic activity was accomplished by one of two procedures. In one, the antiserum, or the globulin fraction of the antiserum (14), was mixed with the thyrotropin preparation, placed at 4° C overnight, and then injected without centrifugation the following morning. In the second procedure the antiserum was injected subcutaneously at 16 hours, and again at 1 hour, prior to the injection of the hormone on the opposite side of the body. Absorption of the antisera with bovine serum did not materially affect the ability of the antisera to neutralize TSH activity. Accordingly, absorption with bovine serum was not routinely performed in these bioassay studies.

RESULTS

Precipitins to TSH and other pituitary hormones analyzed by the Ouchterlony technic. Antisera to all three TSH preparations were found to contain precipitins to bovine serum proteins and yielded one to four bands when tested in the Ouchterlony system. This is illustrated in Figure 1 for two of the antisera to Armour TSH and it is evident that several of the bands of precipitation produced against the homologous TSH preparation are continuous with bands of precipitation produced against bovine serum. Some of the precipitin bands between the antisera and TSH, however, are apparently specific for TSH antigens. These bands are seen near the antibody wells or as spurs (see arrows, Figure 1). Antiserum to Armour TSH after absorption with bovine serum still retains antibody to TSH antigens as shown in Figure 2. Three lines of precipitation are evident between Armour TSH and these two absorbed antisera, whereas all of the precipitins to bovine serum proteins have been removed. The results of the Ouchterlony analyses of several absorbed antisera are summarized in Table I. As noted in this table, antisera to Armour TSH produced two or three lines of precipitation with Armour TSH, two lines with Condliffe TSH, and one or two lines with Pierce TSH preparations. Antisera to Condliffe TSH generally showed two bands of precipitation with the homologous antigen and

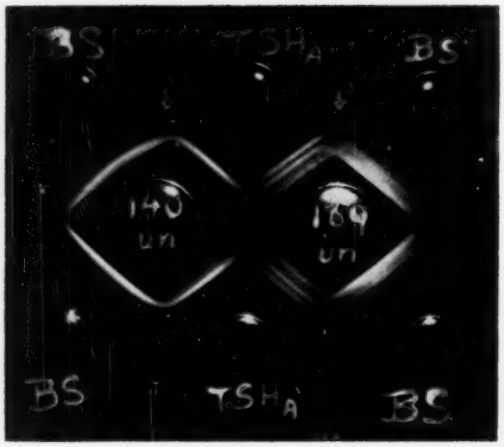

FIG. 1. OUCHTERLONY GEL DIFFUSION ANALYSIS OF THE PRECIPITIN REACTIONS BETWEEN BOVINE SERUM (BS) AND ARMOUR THYROID-STIMULATING HORMONE (TSH_A) AND TWO ANTI-ARMOUR TSH ANTISERA, NOS. 140 AND 169, PRIOR TO ABSORPTION OF THESE ANTISERA WITH BOVINE SERUM. Bands of precipitation produced against TSH_A are continuous with those against bovine serum. An inner band of precipitation and spurs at the site of the arrows (the latter not visible now, following reproduction) are specific for TSH.

with Armour TSH, and one or two bands of precipitation with Pierce TSH. Antisera to Pierce TSH showed one or two bands of precipitation against the homologous antigen and usually only one band when tested with the other two thyrotropins.

Anti-TSH antisera were tested for precipitin reactions with several other hormones of pituitary origin, and these results are presented in Table II. Two preparations of ovine luteinizing hormone (LH) produced precipitation with anti-Armour and anti-Condliffe TSH sera. Figure 3 illustrates the reactions between an anti-Condliffe TSH anti-

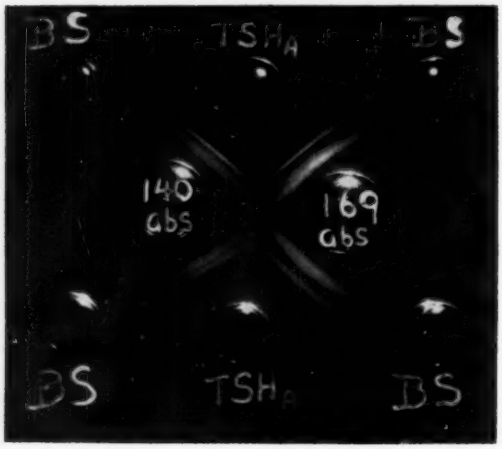

FIG. 2. SAME ANTISERA ILLUSTRATED IN FIGURE I, AFTER ABSORPTION WITH BOVINE SERUM; ONLY THREE BANDS OF PRECIPITATION TO TSH REMAIN.

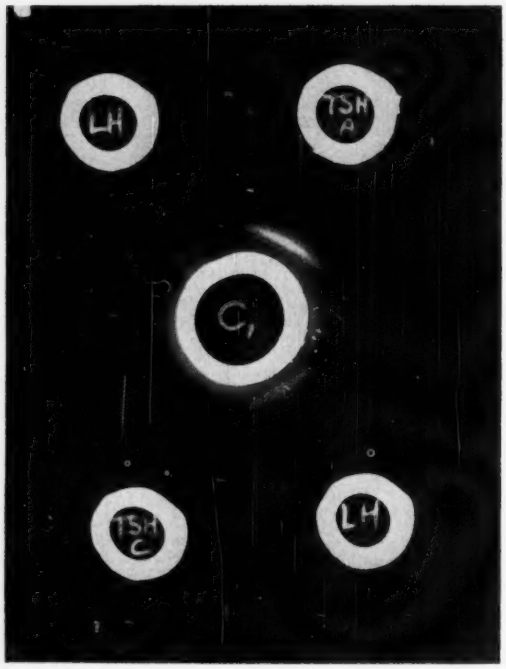

FIG. 3. ANTISERUM TO CONDLIFFE TSH (C_1) IN CENTER WELL; CONDLIFFE TSH, ARMOUR TSH AND WILHELMI LUTEINIZING HORMONE (LH) IN OUTER WELLS. There is a line of precipitation between the antiserum and the luteinizing hormone continuous with a fine band of precipitation to the TSH preparations.

serum (C_1) and two preparations of TSH and LH, respectively. The inner line of precipitation produced by the TSH preparations appears to blend with the band produced by LH, whereas the outer band is apparently specific for a TSH antigen. Figure 4 shows that the C_1 antiserum to Condliffe TSH after absorption with LH still retains a strong precipitin to the TSH antigen and further, as shown in Figure 5, this antigen is common to all three TSH preparations.

TABLE I

Bovine serum absorbed antisera to thyroid-stimulating hormone (TSH) tested by the Ouchterlony technic against homologous and heterologous TSH preparations

Antisera to TSH of	Hormone	Test hormone preparation			Absorbed with bovine serum and	Number of precipitin bands in Ouchterlony plate*
		Source	TSH content (IU/mg)	LH content		
Armour	TSH	Armour	1	++++		2 or 3
Armour	TSH	Condliffe	25	?0		2
Armour	TSH	Pierce	25	?0		1 or 2
Condliffe	TSH	Armour	1	++++		2
Condliffe	TSH	Condliffe	25	see above		2
Condliffe	TSH	Pierce	25	see above		1 or 2
Pierce	TSH	Armour	1	++++		1
Pierce	TSH	Condliffe	25	see above		1
Pierce	TSH	Pierce	25	see above		1 or 2
Armour or Condliffe	TSH	Armour, Condliffe or Pierce	see above	see above	LH	1 or 2 bands
Armour or Condliffe or Pierce	TSH	Armour, Condliffe or Pierce	see above	see above	TSH	0

* Antisera undiluted; solutions of test TSH antigens contained 0.5 or 1.0 IU.

TABLE II

Bovine serum absorbed antisera to TSH tested by the Ouchterlony technic against hormone other than TSH*

Hormone	Test hormone preparation			Absorbed with bovine serum and	Bands in Ouchterlony plate†
	Source	TSH content (IU/mg)	LH content		
LH (ovine)	Wilhelmi	0.1	++++		1 or 2
LH (ovine)	McShan		++++		1 or 2
LH (ovine)	Wilhelmi	0.1	++++	LH	0
LH (ovine)	McShan		++++	LH	0
G.H. (bovine)	NIH	0.008	±		1
G.H. (ovine)	Wilhelmi	0	±		2
G.H. (bovine or ovine)	NIH or Wilhelmi	see above	see above	LH	0
G.H. (human)	Raben	0	0		0
G.H. (human)	Wilhelmi	0	0		0
G.H. (porcine)	Wilhelmi	0	0		0
G.H. (simian)	Wilhelmi	+	+		0
Urine gonadotropin (human)	Albert	0	+		0
Prolactin	NIH	0.004	0		0
Pitressin	Parke, Davis	0	0		0
Pitocin	Parke, Davis	0	0		0
ACTH	Armour	0	0		0

* Antisera to TSH to Armour or Condliffe.
† Antisera undiluted.

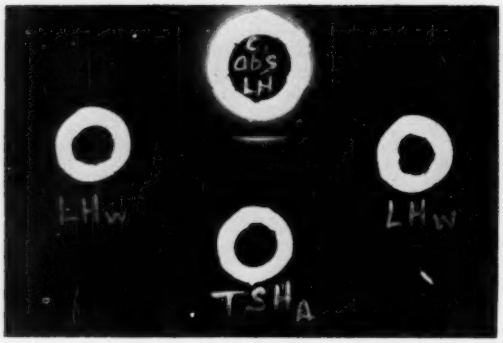

FIG. 4. DEMONSTRATION OF THE PERSISTENCE OF THE REACTIONS BETWEEN TSH AND ANTI-TSH ANTISERUM AFTER ABSORPTION OF THIS ANTISERUM WITH LUTEINIZING HORMONE. Antiserum to Condliffe TSH absorbed with luteinizing hormone (C_1 abs LH) in top well; luteinizing hormone (LHw).

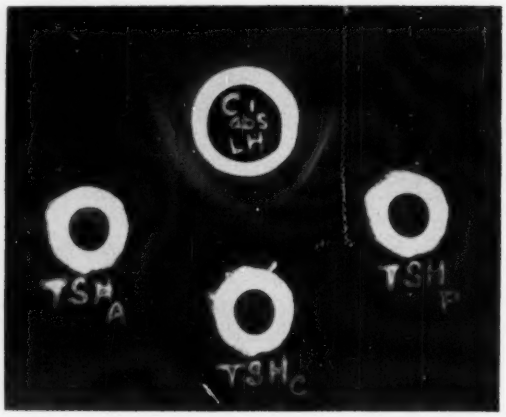

FIG. 5. DEMONSTRATION OF A REACTION OF IDENTITY AMONG THREE TSH PREPARATIONS (ARMOUR, CONDLIFFE AND PIERCE) PRODUCED BY C_1 ABS LH ANTISERUM.

Among the other pituitary hormones tested (Table II) only two preparations of growth hormone gave a band of precipitation with the anti-TSH antisera and this precipitation was eliminated in both instances by absorption with LH, indicating contamination of these GH preparations with LH.

Immunoelectrophoretic analysis. For purposes of estimating the electrophoretic mobilities of the antigens, a reference sample of normal human serum was run on each plate, and reacted with an equine antiserum [1] to pooled normal human serum. These mobility references are illustrated in Figures 8 and 9. It should be noted that, as a result of elctroendosmosis and the flow of buffer toward the cathode during the period of electrophoresis, γ-globulin and part of the β-globulin fraction are displaced to the cathodal side of the antigen well, although their net electrophoretic migrations are in an anodal direction under the conditions of pH 8.6 employed.

The major serum protein constituents present as impurities in the Condliffe and Armour TSH preparations are identifiable by immunoelectrophoretic analysis. In Figure 6A it is seen that a very strong precipitin arc having the characteristic polydispersed electrophoretic distribution of γ-globulin is obtained when anti-Condliffe TSH antiserum is reacted against bovine serum. Figure 6B shows that the major serum protein present in the Armour TSH preparation is albumin. The Armour TSH preparation also contains some γ-globulin, although this is not well seen in Figure 6B. These arcs were eliminated by absorption of these antisera with bovine serum. The reactions of Armour TSH with unabsorbed and absorbed anti-Armour TSH antiserum are shown in Figure 6C. It is seen that the absorption with bovine serum eliminates the precipitin arcs in the albumin and gamma regions, leaving a single arc in the α-2 mobility range.

Figure 7A shows that the anti-Condliffe TSH antiserum, after absorption with bovine serum, produces a single precipitin arc in the fast beta mobility range when reacted with its homologous antigen but, significantly, this same absorbed anti-Condliffe TSH antiserum, when reacted with the Armour TSH preparation, produces three, or possibly four, precipitin arcs covering a broad range of mobility from the slow beta to the fast alpha region (Figure 7B). The details of this reaction between the absorbed anti-Condliffe TSH antiserum and Armour TSH are better illustrated in Figure 8. It is apparent that all of the arcs are immunologically interrelated, as evidenced both by fusion of their extremities and by their similar electrophoretic distributions and geometric shapes. Thus, the inner alpha arc, designated α', has a long cathodal extension traversing into the beta region. The outer alpha arc, designated α'',

[1] Equine antihuman serum no. 511, Institut Pasteur, Paris.

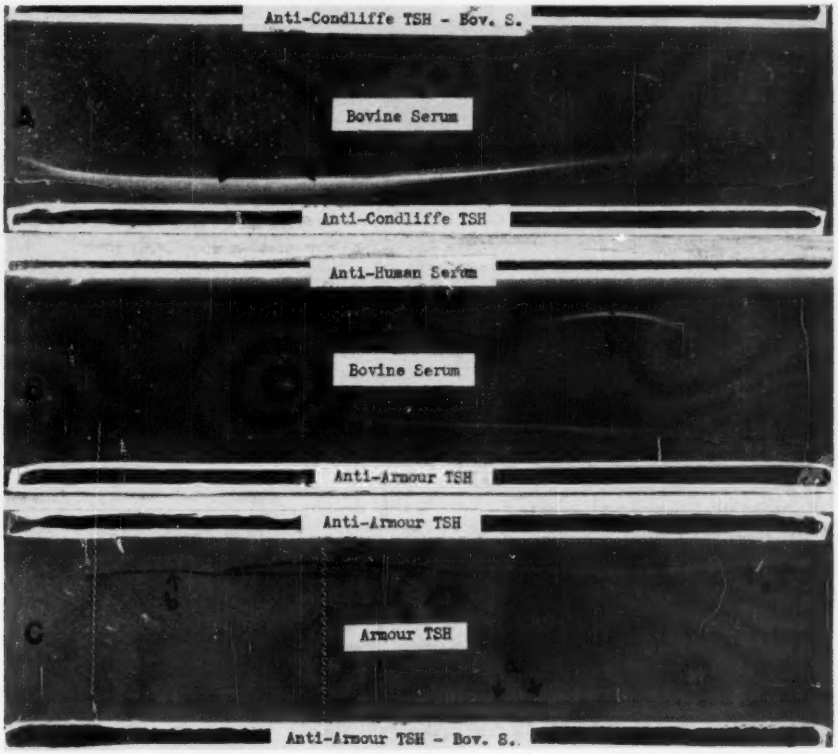

FIG. 6. IMMUNOELECTROPHORETIC ANALYSES OF THE PRECIPITIN REACTIONS OF THE ANTI-TSH ANTISERA PRIOR TO AND FOLLOWING ABSORPTION WITH BOVINE SERUM. In all of these analyses, the direction of electrophoretic migration is from left to right (anode on right); the cathodal displacement of antigens (to the left of the antigen wells) is the result of electroendosmotic flow.

A. REACTION OF BOVINE SERUM WITH UNABSORBED ANTI-CONDLIFFE TSH (LOWER TROUGH) AND ABSORBED ANTI-CONDLIFFE TSH ANTISERA (UPPER TROUGH). The long, polydispersed precipitin arc (arrows) produced by the unabsorbed antiserum has the characteristic distribution and mobility of γ-globulin.

B. REACTION OF BOVINE SERUM WITH UNABSORBED ANTI-ARMOUR TSH ANTISERUM (LOWER TROUGH) DEMONSTRATES A PRECIPITIN ARC AT THE ANODAL END (ARROWS, a) IN THE MOBILITY RANGE OF ALBUMIN. The long cathodal tail of this arc may represent the effect of partial denaturation. A bovine serum protein with the mobility of an α-2 globulin (arrow b) is seen to cross react with an antiserum to normal human serum (upper trough).

C. REACTION OF ARMOUR TSH WITH UNABSORBED (UPPER TROUGH) AND ABSORBED (LOWER TROUGH) ANTI-ARMOUR TSH ANTISERUM. With the unabsorbed antiserum, the arc considered to be that of albumin (arrows a) is again seen, as in B; a faint arc in the gamma mobility range (dotted line, arrow b), and an arc of beta mobility (dashed line, arrow c) are also demonstrated. After absorption with bovine serum, only one arc, of α-2 mobility (arrows, d) remains. (The straight line near the bottom trough is an artifact.)

also can be interpreted as either having a long cathodal extension, or alternately as fusing with another precipitin arc of beta mobility which is designated β'. Finally, the anodal end of the outer beta arc, β'', partially fuses with outer alpha arc, α''. The possible interpretations of these findings are discussed below.

Immunoelectrophoretic analysis of the reaction of luteinizing hormone with anti-TSH-antiserum. As noted above, a preparation of LH of ovine ori-

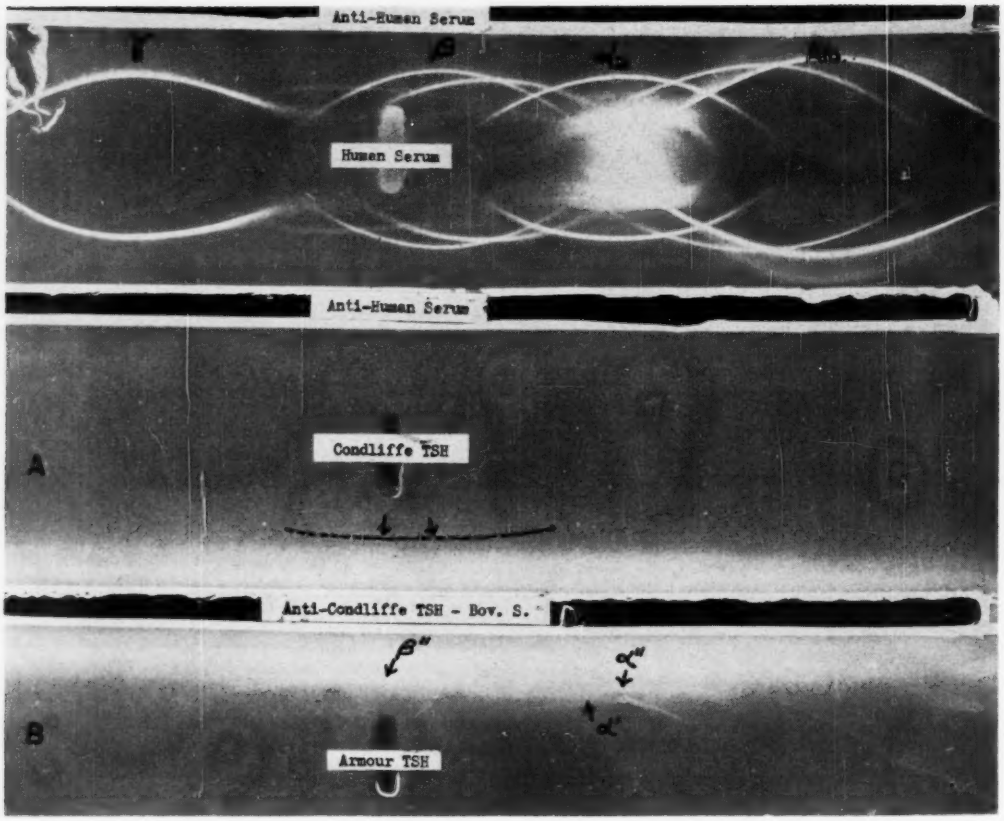

FIG. 7. IMMUNOELECTROPHORETIC ANALYSES OF THE PRECIPITIN REACTIONS BETWEEN THE ARMOUR AND CONDLIFFE TSH PREPARATIONS AND AN ANTI-CONDLIFFE TSH ANTISERUM ABSORBED WITH BOVINE SERUM. For purposes of estimating electrophoretic mobilities, an analysis of human serum reacted with horse antihuman serum is shown in the top section.

A. REACTION OF CONDLIFFE TSH WITH ANTI-CONDLIFFE TSH ANTISERUM ABSORBED WITH BOVINE SERUM (LOWER TROUGH) DEMONSTRATING A SINGLE ARC OF BETA MOBILITY (DOTTED LINE, ARROWS).

B. REACTION OF ARMOUR TSH WITH ANTI-CONDLIFFE TSH ANTISERUM ABSORBED WITH BOVINE SERUM (UPPER TROUGH) DEMONSTRATING A LONG PRECIPITIN ARC WITH APPARENTLY TWO SEPARATE MAXIMA (α'' AND β''), AND ANOTHER ARC OF ALPHA MOBILITY (α'). These arcs are better visualized in Figure 8.

FIG. 8. REACTION OF ARMOUR TSH WITH ANTI-CONDLIFFE TSH ANTISERUM ABSORBED WITH BOVINE SERUM (BOTH TOP AND BOTTOM TROUGHS). See text for discussion of this figure.

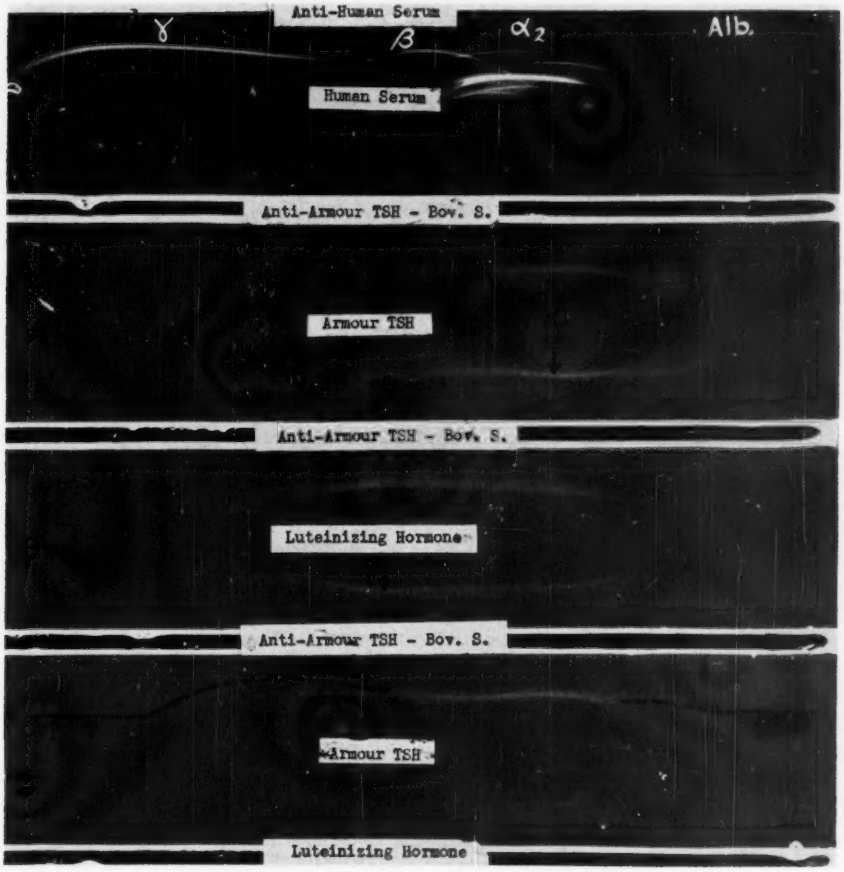

FIG. 9. IMMUNOELECTROPHORETIC ANALYSES OF THE CROSS REACTION BETWEEN OVINE LUTEINIZING HORMONE AND ANTI-ARMOUR TSH ANTISERUM ABSORBED WITH BOVINE SERUM. The upper section shows the reaction of human serum with horse antihuman serum as a mobility reference.

A. REACTION OF ARMOUR TSH WITH ANTI-ARMOUR TSH ANTISERUM ABSORBED WITH BOVINE SERUM (UPPER AND LOWER TROUGHS). A long precipitin arc extending from the fast alpha to the slow beta mobility range is seen; the region of maximal precipitation is in the alpha mobility range.

B. REACTION OF LUTEINIZING HORMONE WITH ANTI-ARMOUR TSH ANTISERUM ABSORBED WITH BOVINE SERUM (UPPER AND LOWER TROUGHS). A long precipitin arc is again seen, but the maximal precipitation is in the beta mobility range.

C. ANALYSIS OF THE INTERRELATIONSHIPS OF ARMOUR TSH AND LUTEINIZING HORMONE, USING A THREE-REACTANT SYSTEM. The Armour TSH was electrophoresed. The antiserum was placed in the upper trough, and the luteinizing hormone in the lower trough. The straight lines of precipitation (dashed lines, arrows) at the anodal and cathodal ends of the agar represent the reaction between the luteinizing hormone and the anti-TSH antiserum. These lines partially fuse with the anodal and cathodal ends of the arc produced by the reaction between the Armour TSH and the antiserum.

gin (Wilhelmi LH) cross reacts with anti-TSH antiserum. The immunoelectrophoretic analysis of this reaction is shown in Figure 9. An anti-Armour TSH antiserum, different from that shown in previous patterns, was employed in this study. When this antiserum was reacted with its homologous antigen (Armour TSH), a strong precipitin arc of alpha mobility and a much weaker

TABLE III

Distribution of TSH activity following electrophoresis in agar; assays performed in I^{131}-labeled, thyroid-suppressed mice

Experiment	No. of mice	Media	% Control radioactivity ±SD at 2 hours
A	4	Control: saline 0.2 cc	95 ± 20
	4	Control: Armour TSH 0.1 mμ	162 ± 17
Strips removed	4	Control: buffered agar	88 ± 12
from the agar	4	Anode agar, 4–5 mm from well	203 ± 67
after 18 hours	4	Anode agar, 2–3 mm from well	250 ± 47
at 4° C	4	Cathode agar 2–3 mm from well	532 ± 44*
	4	Cathode agar, 4–5 mm from well	559 ± 122*
B	4	Control: saline 0.2 cc	91 ± 11
	4	Control: Armour TSH 0.1 mμ	153 ± 9
Strips removed	4	Anode agar, 12–13 mm from well	81 ± 4
from the agar	4	Anode agar, 4–5 mm from well	84 ± 9
immediately	4	Cathode agar, 4–5 mm from well	542 ± 98*
following electrophoresis	4	Cathode agar, 12–13 mm from well	144 ± 23*

* $p < 0.001$.

beta arc (or possibly a beta extension of the alpha arc) was obtained (Figure 9A). When reacted with ovine LH, this antiserum yielded a long arc extending across the beta and alpha regions, but, in contrast to the homologous system, the precipitation maximum of this LH arc was in the beta region (Figure 9B). In an attempt to elucidate the interrelationship of the TSH and LH precipitin arcs, a modified immunoelectrophoretic procedure (15) was utilized whereby the eletrophoretically separated TSH fraction was simultaneously reacted with both the anti-TSH antiserum and the LH (Figure 9C). In this analysis, the LH was permitted to diffuse from a trough in the agar on the side of the antigen reservoir opposite the antiserum trough. This reaction is illustrated in Figure 9C. It is seen that the precipitin reaction between LH and the anti-TSH antiserum is in the form of a straight line, and that the cathodal end of this line partially fuses with the anodal end of the alpha arc of TSH. This establishes a partial immunological relationship between TSH and LH, but not complete identity. The thesis that these proteins are not identical is further supported by the differences in their electrophoretic maxima.

Bioassay for TSH activity following electrophoresis in buffered agar. Two bioassay experiments for TSH activity were performed on agar strips removed from slides following electrophoresis. The results of these experiments are shown in Table III. In the first experiment (A), the slides were kept overnight at 4° C before removal of the agar strips; in the second experiment (B), the agar strips were removed immediately following electrophoresis.

In Experiment A, TSH activity was demonstrable on both the anodal and the cathodal side of the antigen well, corresponding to the distribution of immune precipitation in the control slide developed with anti-TSH antiserum. The activity

TABLE IV

Inhibition of TSH (Armour) activity in the hypophysectomized rat by rabbit antithyrotropin serum

Animals	Amount of Armour TSH	Amount of serum	Nature of rabbit serum	TSH and/or serum administration	Average thyroid cell height
No.	*IU/day*	*ml/day*		*No. of days*	*μ±SD*
6	none	none		hypophysectomized untreated controls	4.3 ± 1.3
3	none	1.0	normal	7	4.5 ± 1.3
6	5	none		7	7.7 ± 1.6
3	5	0.5	normal	7	8.2 ± 1.7
3	5	0.5	anti-Armour TSH	7	5.0 ± 1.3

TABLE V

Inhibition of TSH activity in the mouse by rabbit antithyrotropin serum; assays performed in I^{131}-labeled, thyroid-suppressed mice

Exp't. no.	No. of animals	TSH preparation	Antiserum or globulin fraction	% Control radioactivity ±SD at 2 hours
144	4	None (saline)	None	123 ± 16
	4	Bovine TSH (Armour) 1 mμ	10 mg normal rab. glob. (= 0.5 ml)	310 ± 48
	4	Bovine TSH (Armour) 1 mμ	10 mg anti-Armour glob. (= 0.5 ml)	136 ± 4*
164	4	None (saline)	None	126 = 27
	4	Bovine TSH (Condliffe) 1 mμ	None	230 ± 53
	4	Bovine TSH (Condliffe) 1 mμ	10 mg normal rab. glob. (= 1.0 ml)	217 ± 60
	4	Bovine TSH (Condliffe) 1 mμ	12 mg anti-Armour glob. (= 1.0 ml)	133 ± 19*
214	4	None (saline)	None	151 ± 26
	4	Bovine TSH (Pierce) 1 mμ	None	174 ± 25
	4	Bovine TSH (Pierce) 1 mμ	7.7 mg anti-Pierce glob. (1.0 ml)	104 ± 12*
220	4	None (saline)	None	116 ± 52
	4	Bovine TSH (Pierce) 1 mμ	None	181 ± 60
	4	Bovine TSH (Pierce) 1 mμ	3.85 mg anti-Pierce glob. (= 0.5 ml)	102 ± 19*
220	4	None (saline)	None	85 ± 10
	4	Mouse pit. tumor (Condliffe) 0.5 mμ	None	271 ± 68
	4	Mouse pit. tumor (Condliffe) 0.5 mμ	0.5 ml anti-Armour	106 ± 24
	4	Mouse pit. tumor (Condliffe) 0.5 mμ	1.0 ml anti-Armour	110 ± 28*
226	4	None (saline)	None	85 ± 10
	4	Human TSH (Bakke) 0.25 mμ	None	204 ± 14
	4	Human TSH (Bakke) 0.25 mμ	0.25 ml anti-Armour	111 ± 10*
	4	Human TSH (Bakke) 0.25 mμ	0.5 ml anti-Armour	123 ± 24*
238 and 140	10	None (saline)	None	97 ± 26
	4	Human TSH (Bakke) 0.5 mμ	None	267 ± 133
	4	Human TSH (Bakke) 0.5 mμ	3 mg normal rab. glob. (= 0.25 ml)	337 ± 101
	4	Human TSH (Bakke) 0.5 mμ	2 mg anti-Armour glob. (= 0.1 ml)	113 ± 13*

* $p < 0.001$.

on the cathodal side of the antigen well was considerably greater than on the anodal side. In Experiment B, activity was found only on the cathodal side of the antigen well, suggesting that the activity found on the anodal side in Experiment A was the result, at least in part, of diffusion.

Neutralization of thyrotropin effect by anti-TSH antiserum. By means of the two bioassay systems described above, it was possible to demonstrate that the anti-TSH antisera were capable of neutralizing the thyrotropic actions of both homologous and heterologous TSH preparations. Both the whole antisera and the globulin fractions thereof were effective, whereas normal rabbit serum and its globulin fraction were totally ineffective. A precise titration of the least amount of antiserum which would neutralize a given amount of TSH was not performed, but this is planned for future studies. Table IV presents the results of a bioassay in hypophysectomized rats, employing Armour TSH and anti-Armour TSH antisera. Table V presents the data derived from seven experiments in which antisera to Armour TSH and Pierce TSH were tested for antithyrotropic activity in mice given several different TSH preparations of bovine, human and murine origin. Significant cross reactions between the antisera to bovine TSH and the thyrotropin preparations obtained from other species are evident, but precise quantitative data were not obtained due to the lack of availability of sufficient amounts of the human and murine TSH preparations.

DISCUSSION

In 1958, Henry and Van Dyke (16) described the production and characterization of precipitating antibodies to bovine luteinizing hormone, and in 1959 Hayashida and Li (17) reported similar immunochemical studies of antisera to pituitary growth hormone of various species. The present report describes the results of an immunologic and biologic analysis of antisera to bovine thyrotropin preparations. Precipitating antibody was produced, and this antibody appears to be specific for bovine thyrotropic hormone. A reaction of identity as defined by the criteria of Ouchterlony (10) was obtained when the anti-TSH antiserum was tested against three different preparations of bovine TSH of varying degrees of purity.

In addition to the precipitins to TSH, these antisera were demonstrated to contain precipitating antibodies to various bovine serum proteins and also to LH. By the immunoelectrophoretic technic, it was possible to identify albumin and γ-globulin as the major serum protein contaminants of the Armour TSH preparation, and γ-globulin as the principal contaminant of the more highly purified TSH preparation of Condliffe. After absorption of the antisera with bovine serum, these precipitins were removed, leaving only the precipitins to TSH and to LH. Additional absorption of the anti-TSH antisera with LH removed the precipitins to LH, leaving only the precipitins to TSH. Significantly, the precipitin reaction which had been observed between the anti-TSH antisera and two preparations of pituitary growth hormone, prior to the absorption of the antisera with LH, was no longer present after absorption. This observation has been interpreted as indicating contamination of these growth hormone preparations with luteinizing hormone.

Whereas the immunoelectrophoretic analysis of the reaction between the highly purified Condliffe TSH preparation and its homologous antiserum revealed a single precipitin arc in the beta mobility range, the reaction between this same antiserum (anti-Condliffe TSH) and the Armour TSH preparation revealed a uniquely complex pattern with four precipitin arcs; two of alpha and two of beta mobility. All four of these arcs appeared to be immunologically interrelated as evidenced both by fusion of their extremities and by their similar electrophoretic distributions and geometric shapes. There would appear to be at least three possible interpretations of these immunoelectrophoretic findings. *1*) There may be two immunologically related, but electrophoretically distinct proteins in the Armour TSH preparations, one of alpha and one of beta mobility, and the reduplication of the arcs may represent the effect of antigen or antibody excess as elucidated by Korngold and van Leeuwen (18). *2*) There may be two distinct proteins (alpha and beta), and the reduplication of their respective arcs may be the result of partial denaturation or degradation, as detailed in the studies of Lapresle (19). *3*) Each of the arcs may truly represent a separate and distinct protein constituent. It is impossible to choose among these alternatives on the basis of the present data.

Confirmation of the fact that TSH migrates in an electrical field was obtained by the bioassay of segments of buffered agar following electrophoresis. The maximal thyrotropic activity was found in the beta mobility range and corresponded to the region of maximal immune precipitation. It should be re-emphasized that the cathodal displacement of this arc (and of the active thyrotropic hormone) is the result of electroendosmotic flow, and that an uncharged moiety would have been displaced much farther toward the cathode under these conditions of electrophoresis in agar. It is noteworthy that recent studies by Carsten and Pierce (20) of chromatographically purified preparations of TSH have demonstrated migration of TSH activity in starch gel electrophoresis over a wide pH range, including pH 8.6 used in the present studies. It is not possible, however, to compare the electrophoretic mobility of a protein in agar with its mobility in the starch gel medium, since these media, per se, exert unique physical influences on the movement of proteins through them.

A partial immunologic relationship has been shown to exist between thyrotropin and LH but immunoelectrophoretic analysis has established that these two pituitary principles are not immunochemically identical, and non-identity is further supported by the observed differences in the electrophoretic maxima of the two hormones.

By two different bioassay methods, the anti-TSH antisera have been shown to be capable of neutralizing the thyrotropic activity of both homologous and heterologous TSH preparations. Cross neutralization of TSH activity was demonstrated between the anti-bovine TSH antisera and TSH from mouse pituitary tumor and human pituitary sources. Thus, antisera to TSH would seem not to be as highly species specific as antisera to growth hormone (17), and this is in good correspondence with the known effectiveness of TSH from bovine and other sources in heterologous species.

It is still not established whether the precipitating antibody to beef TSH is directed to and specific for the hormonally active groupings of the TSH molecule or to some other part of the molecule. As stated previously, studies of flavianic acid preparations of TSH (4) have suggested that the biologically active part of the thyrotropin molecule may not be antigenic. If, indeed, the antibodies are directed against a physiologically inactive part of the TSH molecule, the neutralizing ability of the antisera might be explained as due to the complexing of thyrotropin by antibody, and sequestration of this complex in the reticuloendothelial system.

SUMMARY

1. Antisera to three bovine thyrotropin preparations were produced by hyperimmunization of rabbits, and these antisera were immunologically and biologically characterized.

2. Precipitating antibody, apparently specific for thyrotropin, was demonstrated by gel diffusion technics.

3. Parallel immunoelectrophoretic analyses and bioassays for thyroid-stimulating hormone (TSH) activity of segments of agar after electrophoresis confirmed the migration of thyrotropin in an electrical field and support the conclusion that the precipitins are specific for the thyrotropic principle.

4. Luteinizing hormone was found to be immunologically related to, but not identical with thyrotropin.

5. By two different bioassay methods, the anti-TSH antisera were shown to be capable of neutralizing the thyrotropic activity of both homologous and heterologous TSH preparations.

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THE EFFECT OF ACETYLCHOLINE UPON RESPIRATORY GAS EXCHANGE IN MITRAL STENOSIS

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Most patients with mitral stenosis show hemodynamic evidence of an increased resistance to blood flow through the lungs. Structural abnormalities in the small pulmonary arteries are seen in such patients, with increased thickness of the vessel wall and narrowing of the lumen. The increased resistance is also in part due to active vasoconstriction in many patients, and this can be released by acetylcholine (1–3). Söderholm and Werkö (4) also observed that the oxygen saturation of arterial blood decreased during the infusion of acetylcholine in patients with mitral stenosis. They suggested that this was probably due to the increased perfusion of poorly ventilated alveoli which had previously been poorly perfused.

The present studies were undertaken in order to investigate the above possibility further by the measurement of blood gas tensions. Observations have been made while the patients breathed a gas mixture containing from 45 to 50 per cent oxygen, this level of oxygen being selected for two reasons. Firstly, the magnitude of the difference between alveolar and arterial oxygen tension (A-a difference) is greater, and hence it can be measured with a relatively greater precision. Secondly, with this concentration of inspired oxygen the "membrane component" is negligible. A subsidiary purpose of the study was the comparison of the pulmonary hemodynamics in these patients when they breathed air and when they breathed 45 to 50 per cent oxygen.

METHODS

All the patients had rheumatic heart disease with mitral stenosis, and their personal details are given in Table I. Patients were studied in the supine position, unsedated and having taken no food since a light breakfast 5 hours earlier.

A double-lumen cardiac catheter was passed into the lung so that the terminal orifice was wedged in the periphery while the proximal orifice lay in the pulmonary artery. The tip of another single-lumen catheter was introduced into the middle of the right atrium. An indwelling needle was placed in the brachial artery. Intravascular pressures were recorded by capacitance manometers and a direct writing recorder. Mean pressures were obtained by planimetric integration over at least 2 respiratory cycles.

The percentage oxygen saturation of blood samples was estimated by a spectrophotometric method (5), and the blood oxygen capacity was measured photometrically. Gas analysis was by the Scholander micro-method. Inspired gas mixtures other than air were supplied from cylinders, the patient breathing from an anesthesia bag which served as a reservoir.

The oxygen tension of arterial blood was measured polarographically with a Clark electrode incorporated in an assembly with a magnetic stirrer (6). Each reported value of oxygen tension represents the mean of estimates on 3 blood samples. Carbon dioxide tension was measured by a modification of the interpolation method of Åstrup (7) using a Metrohm EA 125 glass electrode. All measurements were made in a water bath at 38° C. Two syringes of blood were centrifuged at 38° C and a reading of plasma pH was made on each. The plasma was then equilibrated by bubbling with 3 gas mixtures of known carbon dioxide content and the resulting pH values recorded. The initial plasma P_{CO_2} was determined graphically from the relationship between pH and P_{CO_2}. Using this method in 16 tonometer experiments, the mean difference between measured and tonometer values was 0.6 mm Hg (SD 1.2).

The same protocol was followed 3 times in each patient with intervals of 15 minutes between each. In one, the patient breathed air and in another a previously analyzed mixture containing from 45 to 50 per cent oxygen in nitrogen (subsequently referred to as 47 per cent oxygen). On the other occasion the patient breathed the same oxygen-enriched gas mixture but at Minute 11 an infusion of acetylcholine into the right atrium was commenced at the rate of 2.0 mg per minute and continued to the end of the protocol. The 6 permutations of these 3 protocols were placed in random order and allocated to the patients in the order in which they were studied. The 6 experimental plans were then repeated in the same random order, a total of 12 patients being investigated.

Each protocol lasted 25 minutes and the mouthpiece and noseclip were in place throughout. Expired gas was collected and 3 samples of blood from the brachial and

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TABLE I

Personal and clinical details of patients studied *

Patient	Age	Sex	Weight	Height	SA	Rhythm	Valvular lesions		Other clinical features
	yrs		*kg*	*cm*	m^2				
F.A.	58	F	54	155	1.51	AF	MS	MI	
J.Ly.	49	M	66	168	1.71	SR	MS		
D.D.	25	F	63	165	1.68	SR	MS	AI	
E.R.	37	F	60	163	1.63	AF	MS	MI	Recurrent bronchitis
C.W.	25	F	57	160	1.58	SR	MS		Pulmonary infarct 2 months earlier
D.R.	40	F	57	163	1.58	SR	MS		Mitral valvotomy 4 years earlier; recurrent bronchitis
L.M.	48	F	48	155	1.24	AF	MS	MI	Mitral valvotomy 5 years earlier; recurrent bronchitis
M.McP.	45	F	59	155	1.57	SR	MS		Splenectomy for acholuric jaundice 2 years earlier
P.K.	30	F	50	163	1.51	AF	MS	MI	
E.M.	49	M	62	173	1.72	SR	MS		Recurrent bronchitis
J.La.	49	M	79	170	1.90	SR	MS		Mitral valvotomy 4 years earlier; probable pulmonary infarct 1 year earlier
N.T.	46	F	48	163	1.50	AF	MS	MI	Recurrent bronchitis

* Abbreviations used: SA = surface area; AF = auricular fibrillation, SR = sinus rhythm; MS = mitral stenosis, MI = mitral insufficiency, AI = aortic insufficiency.

pulmonary arteries were taken for estimation of the cardiac output between Minutes 15 and 18. Exercise on a constant rate bicycle ergometer commenced at Minute 19 and the cardiac output was again measured between Minutes 23 and 25. Intravascular pressures were recorded immediately before and after each estimation of cardiac output. In each of the 2 protocols in which the high oxygen mixture was breathed, but not in the air-breathing protocol, arterial blood was also taken during the expired gas collection for estimation of gas tensions. Three samples for oxygen tension and two for carbon dioxide tension were taken on each occasion, and sampling extended over most of the period of gas collection.

In calculating cardiac output when oxygen-enriched mixtures were breathed, the arterial oxygen content was taken as equal to the blood oxygen capacity, plus the oxygen in physical solution, which was obtained from the measured oxygen tension and the solubility coefficient of oxygen in blood (8). In a small number of instances this procedure may have led to a slight overestimate of the arterial oxygen content when the arterial oxygen tension was below 150 mm Hg, since knowledge of the form of the hemoglobin oxygen dissociation curve at oxygen tensions greater than 100 mm Hg is inadequate to permit the necessary correction for a possible slight unsaturation. Mixed venous blood oxygen content was calculated from the measured oxygen saturation and the blood oxygen capacity. The alveolar oxygen tension was calculated from the alveolar equation. Physiological dead space was estimated in the usual manner, allowance being made for an apparatus dead space of 80 ml.

The significance of differences between values when breathing air and when breathing 45 to 50 per cent oxygen, and between values when breathing 45 to 50 per cent oxygen with and without the infusion of acetylcholine, have been determined by the use of the *t* test (Tables II and III).

RESULTS

The results are given in Tables II and III and will be described in two sections. The first concerns the observations on blood and alveolar gas tensions, and the second comprises the hemodynamic and related measurements.

I. Arterial blood and alveolar gas tensions and derived measurements

Control values at rest, breathing 47 per cent oxygen. The mean alveolar oxygen tension was 291 mm Hg (range 277 to 316), the mean arterial

TABLE II

Cardiac output and intravascular pressures at rest (R) and during exercise (E) *

Patient		$\dot{V}_{O_2}$		$Ca_{O_2}-C\bar{v}_{O_2}$		$\dot{Q}$		Heart rate		Mean pressure: Pulmonary artery		Mean pressure: Pulmonary wedge		Mean pressure: ΔP		Mean pressure: Brachial artery	
		R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E
		ml/min STPD		*ml/100 ml*		*L/min*		*beats/min*		*mm Hg*		*mm Hg*		*mm Hg*		*mm Hg*	
F.A.	Air	233	399	6.1	8.7	3.8	4.6	132	149	39	56	22				138	148
	O_2	246	420	5.5	8.9	4.5	4.8	122	137	53	57		34			118	133
	ACh	225	437	5.4	9.2	4.2	4.8	109	129	28	43	26				140	148
J.Ly.	Air	236	903	4.1	9.5	5.7	9.5	79	144	33	94	20	45			98	124
	O_2	218	726	4.6	9.5	4.7	7.7	95	140	23	58					88	114
	ACh	196	744	4.4	9.5	4.5	7.9	89	134	25	60	18	42			100	126
D.D.	Air	227	744	3.9	8.5	5.8	8.7	86	142	34	69	25	46			94	102
	O_2	300	834	4.0	9.0	7.5	9.2	80	137	29	63	22	49			92	104
	ACh	307	808	3.7	8.2	8.3	9.9	84	129	33	61	30				98	110
E.R.	Air	217	791	5.1	12.3	4.3	6.4	88	138	30	46	20	28	10	18	110	119
	O_2	214	759	5.2	11.9	4.1	6.4	84	124	23	38	14	28	9	10	99	111
	ACh	208	774	5.4	11.3	3.9	6.9	79	120	24	42	16	31	8	11	105	106
C.W.	Air	226	439	5.2	7.5	4.3	5.9	119	152	41	72	19	21	22	51	75	81
	O_2	204	419	4.6	7.5	4.4	5.6	105	128	34	60	20	31	14	29	75	80
	ACh	209	435	4.8	7.8	4.4	5.6	109	128	32	49	17	31	15	18	67	70
D.R.	Air	224	616	6.0	13.9	3.8	4.4	75	160	57	91	22	30	35	61	69	
	O_2	229	563	6.4	14.6	3.6	3.9	67	147	47	86	21	26	26	60	83	90
	ACh	222	649	6.0	14.5	3.7	4.5	75	154	50	97	25	26	25	72	85	88
L.M.	Air	191	356	4.4	7.1	4.4	5.0	94	115	37	58	20	33	17	25	135	144
	O_2	238	331	5.0	6.0	4.8	5.5	99	113	53	55	27	33	26	22	134	144
	ACh	214	423	4.1	6.1	5.2	6.9	103	113	34	45	23	34	11	11	137	148
M.McP.	Air	180	690	4.4	9.8	4.1	7.1	72	136	21	39	13	29	8	10	91	112
	O_2	210	670	4.8	9.8	4.4	6.8	87	128	15	32	9	25	6	7		
	ACh	204	696	4.7	9.8	4.4	7.1	75	131	18	37	12	29	6	8		
P.K.	Air	196	411	6.2	9.8	3.2	4.2	77	102	46	78					89	99
	O_2	241	518	5.8	10.0	4.2	5.2	72	97	41	74					96	104
	ACh	238	485	5.3	9.7	4.5	5.0	73	92	48	73					97	109
E.M.	Air	259	777	4.8	11.3	5.4	6.9	73	119	47	98					104	122
	O_2	253	742	4.1	10.6	6.2	7.0	74	120	40	87					99	102
	ACh	250	728	4.3	10.4	5.8	7.0	71	112	36	74					95	108
J.La.	Air	278	638	5.2	8.5	5.4	7.6	75	94	28	54	19	39	9	15	97	104
	O_2	302	738	4.6	8.1	6.5	9.1	77	90	31	58	21	36	10	22	99	107
	ACh	330	670	4.6	7.9	7.3	8.5	74	91	30	58	25	48	5	10	95	116

* Abbreviations as recommended (9) with the following additions: Air, breathing air; O_2, breathing 45 to 50% oxygen; ACh, breathing 45 to 50% oxygen, during infusion of acetylcholine; ΔP, mean pulmonary arterial pressure − mean pulmonary wedge pressure.

TABLE II—(*Continued*)

Patient		$\dot{V}_{O_2}$		$Ca_{O_2}-C\bar{v}_{O_2}$		$\dot{Q}$		Heart rate		Mean pressure							
										Pulmonary artery		Pulmonary wedge		ΔP		Brachial artery	
		R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E
		ml/min STPD		*ml/100 ml*		*L/min*		*beats/min*		*mm Hg*		*mm Hg*		*mm Hg*		*mm Hg*	
N.T.	Air	226	419	7.1	11.3	3.2	3.7	96	141	55	74	17	27	28	47	133	133
	O_2	203	422	7.4	11.7	2.8	3.6	90	128	46	70	24	28	22	42	134	138
	ACh	220	467	7.3	12.5	3.0	3.7	82	137	65	71	27	25	38	46	136	144
Mean	Air	224.4	598.6	5.21	9.84	4.44	6.17	88.8	132.6	37.9	68.8	18.2†	29.4	18.6†	32.4	107.3‡	117.6
	O_2	238.2	595.2	5.16	9.79	4.81	6.23	87.7	124.1	35.9	61.3	19.3	29.4	15.9	27.4	103.4‡	113.7
	ACh	235.3	609.7	4.99	9.72	4.92	6.48	85.3	122.5	34.9	58.9	20.6	31.6	15.1	25.1	107.0	118.5
Air-O_2	/§	1.552	0.151	0.320	0.290	1.685	0.239	0.432	4.314	0.777	2.604	0.565	0.040	1.110	1.516	1.574	1.398
O_2-ACh	t	0.204	1.068	1.610	0.595	0.971	1.885	1.243	0.931	0.311	1.045	1.006	1.267	0.200	0.648	1.310	2.050

† Mean values for pulmonary wedge and ΔP for 7 patients only.
‡ Mean values for brachial arterial pressure for 10 patients only.
§ Student's t test.

blood oxygen tension was 200 mm Hg (range 142 to 275), and the mean A-a difference was 92 mm Hg (range 39 to 138).

The mean arterial carbon dioxide tension was 39.7 mm Hg (range 32.1 to 45.2). The mean value for physiological dead space was 123 ml with a range from 78 to 208. The physiological dead space/tidal volume ratio (V_D/V_T) had a mean value of 30.0 per cent (range from 21.2 to 40.9).

Effect of exercise, breathing 47 per cent oxygen. The mean alveolar oxygen tension was not appreciably different from the value at rest, and the greatest individual change was 10 mm Hg. The mean arterial oxygen tension was slightly but insignificantly less than at rest. The resultant A-a difference had a mean value which was a little greater than at rest.

The mean arterial carbon dioxide tension rose to 41.8 mm Hg during exercise. The physiological dead space increased considerably to a mean of 190 ml, but there was only a small increase in the ratio V_D/V_T, which had a mean value of 31.3 per cent.

The changes in A-a difference and in the ratio V_D/V_T during exercise varied in the individual patients. In general, both changed in the same direction in a particular patient and both tended to increase more consistently in patients with the higher pulmonary arterial pressures. The ratio V_D/V_T tended to increase to a greater extent in patients in whom this value at rest exceeded 30 per cent.

Effect of acetylcholine at rest. The infusion of acetylcholine resulted in no significant alteration in the mean values of alveolar or arterial oxygen tension, and the mean A-a difference was not appreciably different. Arterial carbon dioxide tension was unchanged and physiological dead space and the ratio V_D/V_T were not significantly altered by acetylcholine.

Effect of acetylcholine during exercise. The mean alveolar oxygen tension was similar to the mean values at rest and during exercise without acetylcholine. In contrast to the effect of exercise alone, the arterial oxygen tension rose slightly during exercise with acetylcholine, although the difference was not significant. The mean A-a difference therefore decreased slightly instead of increasing

TABLE III

Pulmonary ventilation, blood gas tensions and derived values at rest (R) and during exercise (E) *

Patient		$\dot{V}_E$		f		V_T		R		P_{aCO_2}		P_{AO_2}		P_{aO_2}		$P_{AO_2}-P_{aO_2}$		V_D Physiol.		V_D/V_T	
		R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E
		L/min (BTPS)		*breaths/min*		*ml (BTPS)*				*mm Hg*		*mm Hg*		*mm Hg*		*mm Hg*		*ml BTPS*		%	
F.A.	Air	6.8	10.8	14	20	487	538	0.75	0.72												
	O_2	6.1	9.2	13	18	472	514	0.68	0.64	37.5	40.7	291	285	219	179	72	106	100	121	21.2	23.6
	ACh	5.5	9.6	12	17	460	564	0.63	0.62	37.9	39.7	285	286	162	186	123	100	115	139	25.0	24.7
J.Ly.	Air	7.5	30.7	18	28	410	1078	0.78	0.98												
	O_2	8.6	28.2	20	28	425	989	0.97	1.26	40.5	40.9	314	319	275	262	39	57	124	230	29.2	23.3
	ACh	7.5	26.5	18	30	410	870	0.90	1.14	40.5	42.4	313	316	242	267	71	49	124	225	30.2	25.8
D.D.	Air	8.6	24.7	23	34	375	725	0.80	0.87												
	O_2	8.8	22.2	23	36	382	616	0.61	0.70	32.1	32.4	316	319	246	262	70	57	92	110	24.1	17.8
	ACh	8.1	21.3	17	29	479	734	0.54	0.61	34.2	34.3	309	313	180	248	129	65	157	231	32.8	31.5
E.R.	Air	8.1	29.8	24	46	336	642	0.82	0.93												
	O_2	8.4	25.8	27	41	308	624	0.78	0.93	35.1	40.4	294	293	241	243	53	50	78	181	25.3	29.0
	ACh	8.7	23.0	27	37	320	615	0.85	0.84	36.8	43.7	294	287	230	223	64	64	89	191	27.8	31.0
C.W.	Air	7.2	14.3	20	35	358	409	0.76	0.82												
	O_2	6.9	13.4	20	30	346	448	0.78	0.81	45.2	46.6	277	277	217	199	60	78	115	159	33.2	35.5
	ACh	7.1	13.0	21	33	338	393	0.75	0.73	44.9	47.6	276	272	204	198	72	74	115	139	34.0	35.4
D.R.	Air	10.0	30.8	25	38	393	796	0.79	0.95												
	O_2	9.7	22.8	23	30	416	749	0.75	0.88	32.2	39.3	284	280	200	172	84	108	138	310	33.2	41.4
	ACh	10.7	24.6	27	31	388	782	0.81	0.90	30.6	37.8	287	282	234	210	53	72	125	280	32.2	35.8
L.M.	Air	5.8	9.3	15	20	284	460	0.82	0.76												
	O_2	6.0	8.0	16	18	369	442	0.70	0.74	45.2	44.6	278	282	142	155	136	127	92	103	25.0	23.3
	ACh	5.9	8.2	15	20	389	408	0.72	0.53	41.5	41.8	281	274	156	169	125	105	98	97	25.2	23.8
M.McP.	Air	5.9	22.3	17	30	345	736	0.84	0.96												
	O_2	5.9	21.4	20	36	323	590	0.70	0.91	42.2	39.2	294	304	156	135	138	169	93	137	28.8	23.2
	ACh	6.0	21.5	19	33	315	646	0.72	0.88	41.0	39.7	296	303	143	146	153	157	74	164	23.5	25.4
P.K.	Air	6.9	17.9	17	33	402	539	0.74	0.87												
	O_2	8.1	15.6	19	32	424	483	0.77	0.65	36.6	38.5	283	276	234	220	49	56	115	169	27.1	35.0
	ACh	6.5	16.5	17	34	378	482	0.64	0.75	39.7	39.2	274	280	247	225	27	55	104	167	27.5	34.6
E.M.	Air	9.8	31.9	27	51	356	610	0.74	0.82												
	O_2	9.3	27.1	21	51	433	519	0.79	0.79	43.6	46.3	278	275	143	181	135	168	169	228	39.0	44.0
	ACh	10.4	26.1	27	54	375	471	0.76	0.76	43.1	47.5	277	272	109	171	94	101	157	209	41.9	44.3

* See Footnotes to Table II.

TABLE III—(*Continued*)

Patient		$\dot{V}_E$		f		V_T		R		Pa_{CO_2}		P_{AO_2}		Pa_{O_2}		$P_{AO_2}-Pa_{O_2}$		V_D Physiol.		V_D/V_T	
		R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E
		L/min (BTPS)		*breaths/min*		*ml (BTPS)*				*mm Hg*		*mm Hg*		*mm Hg*		*mm Hg*		*ml BTPS*		%	
J.La.	Air	8.1	24.2	17	34	475	711	0.80	0.87												
	O_2	9.6	21.1	19	30	508	702	0.70	0.71	43.5	46.6	305	302	171	216	134	86	208	298	40.9	42.4
	ACh	9.8	20.9	20	30	492	696	0.66	0.76	43.2	51.7	303	298	236	245	67	53	194	334	39.4	48.0
N.T.	Air	7.0	14.9	16	25	436	590	0.76	0.83												
	O_2	6.6	12.3	14	19	466	641	0.82	0.78	43.2	46.0	281	276	150	127	131	149	152	239	32.6	37.3
	ACh	6.5	12.7	14	18	458	701	0.76	0.77	43.5	42.8	278	279	175	143	103	136	144	222	31.4	31.7
Mean	Air	7.64	21.80	19.4	32.8	396.4	652.8	0.784	0.864												
	O_2	7.88	18.93	19.6	30.8	406.0	609.8	0.753	0.817	39.7	41.8	291.3	290.7	199.5	195.9	91.8	100.9	123.0	190.4	30.0	31.3
	ACh	7.73	18.66	19.5	30.5	400.2	613.5	0.726	0.774	39.7	42.4	289.4	288.5	193.2	202.6	90.1	85.9	124.7	199.8	30.9	32.7
Air-O_2	t	1.142	4.989	0.220	1.870	1.101	2.540	1.050	1.325												
O_2-ACh	t	0.695	0.768	0.087	0.278	0.519	0.203	1.658	1.815												

a little as occurred during exercise without acetylcholine. Arterial carbon dioxide tension increased with exercise and the increase was similar to that without acetylcholine. Physiological dead space and the V_D/V_T both increased with exercise to a slightly but insignificantly greater extent than before the infusion of acetylcholine.

Correlations between derived measurements. Since there were no significant mean differences when the group of patients as a whole was considered, the data were inspected to see whether any patterns of response could be found.

A. Correlations between control values at rest. The physiological dead space increased with increasing pulmonary arterial pressure (Figure 1), but the correlation coefficient was insignificant in the present data. When data were added from an additional 12 patients, studied previously under similar conditions for another purpose, this value reached the significant value of 0.5558 ($p < 0.01$).

There was no close correlation between the A-a difference and the physiological dead space at rest ($R = 0.4571$), although larger A-a differences tended to be associated with higher values of dead space. There was no detectable correlation between A-a difference and pulmonary arterial pressure, and when data from the further 12 patients were added the correlation was only slightly improved.

B. Correlations between changes in A-a difference, physiological dead space and other measurements during the infusion of acetylcholine at rest. The relationship of the change in A-a difference with acetylcholine to the A-a difference at rest without acetylcholine (the latter subsequently referred to as the control value) is shown in Figure 2. Patients with a large control value tended to have a decrease with acetylcholine, while patients with a smaller control value showed an increase. Similarly, as shown in Figure 3, a large value of V_D/V_T before the infusion tended to be associated with a decrease of A-a difference with acetylcholine, while the reverse occurred in patients with a lower value of V_D/V_T ($R = 0.7944$, $p < 0.01$). There was a tendency for the A-a difference to decrease during the infusion of acetylcholine in the patients with higher pulmonary arterial pressures, while patients with lower pressures showed an increased A-a difference (Figure 4; $R = 0.3232$).

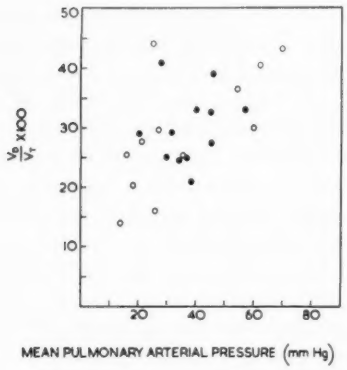

FIG. 1. THE RATIO $(V_D/V_T) \times 100$ PLOTTED AGAINST THE MEAN PULMONARY ARTERIAL PRESSURE. The measurements of $(V_D/V_T) \times 100$ were made at rest, during 47 per cent oxygen breathing. The measurements of pulmonary arterial pressure were made at rest, breathing air. The closed circles represent the patients studied in the present series. The open circles represent an additional 12 patients studied under similar conditions (see text).

The physiological dead space tended to increase during the infusion of acetylcholine when the control value for V_D/V_T was small and to decrease when this value was larger, as shown in Figure 5. Physiological dead space tended to increase with acetylcholine in patients with smaller control values for A-a difference and tended to decrease in patients with larger control values for A-a difference, (Figure 6, $R = 0.3612$). The correlation between pulmonary arterial pressure and the change in V_D with acetylcholine was poor, although there was a tendency for patients with

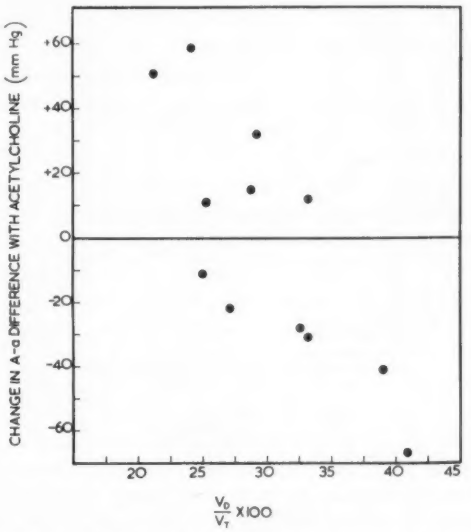

FIG. 3. CHANGE IN A-a DIFFERENCE DURING THE INFUSION OF ACETYLCHOLINE PLOTTED AGAINST THE RATIO $(V_D/V_T) \times 100$ MEASURED BEFORE THE INFUSION. All measurements taken at rest, breathing 47 per cent oxygen.

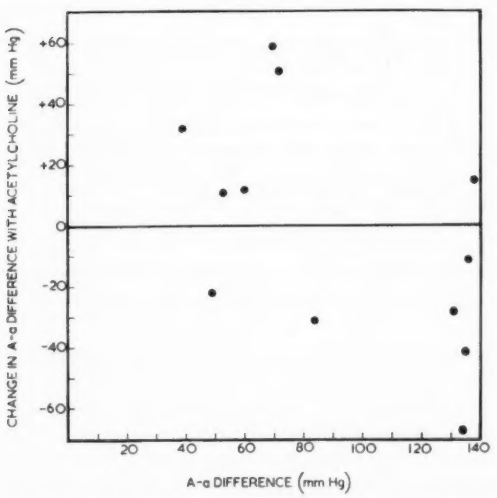

FIG. 2. CHANGE IN A-a DIFFERENCE DURING THE INFUSION OF ACETYLCHOLINE PLOTTED AGAINST THE A-a DIFFERENCE MEASURED BEFORE THE INFUSION. All measurements taken at rest, breathing 47 per cent oxygen.

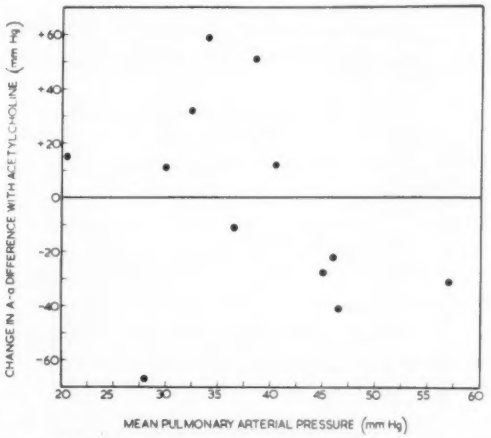

FIG. 4. CHANGE IN A-a DIFFERENCE DURING THE INFUSION OF ACETYLCHOLINE PLOTTED AGAINST THE MEAN PULMONARY ARTERIAL PRESSURE. The measurements of A-a difference were taken at rest, breathing 47 per cent oxygen. The measurements of pulmonary arterial pressure were taken at rest, breathing air.

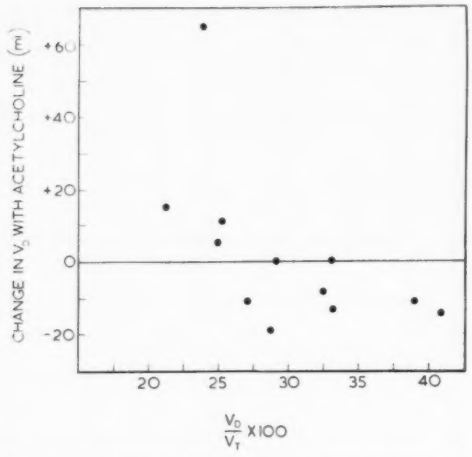

FIG. 5. CHANGE IN PHYSIOLOGICAL DEAD SPACE DURING THE INFUSION OF ACETYLCHOLINE PLOTTED AGAINST THE RATIO $(V_D/V_T) \times 100$ MEASURED BEFORE THE INFUSION. All measurements taken at rest, breathing 47 per cent oxygen.

higher pressures to show a decrease in dead space during infusion, while most patients with lower pressures showed an increase.

The changes in physiological dead space and A-a difference are illustrated in Figure 7, where it is clear that both tend to increase or decrease together under the influence of acetylcholine ($R = 0.7020$, $p < 0.02$).

C. Correlations between changes in A-a difference, physiological dead space and other measurements during the infusion of acetylcholine during exercise. Essentially similar correlations were found when acetylcholine was infused during exercise as were found when it was infused at rest. The A-a difference, which decreased in the group as a whole, tended to decrease to a greater extent in those patients with a high resting value for pulmonary arterial pressure, V_D/V_T and A-a difference. Physiological dead space tended to decrease in patients with the higher pulmonary arterial pressures and to increase in patients in whom pressure was less elevated. Physiological dead space tended to increase in patients in whom the value at rest was normal and to decrease in patients with high resting values. There was no correlation between the change in physiological dead space and the resting value for A-a difference.

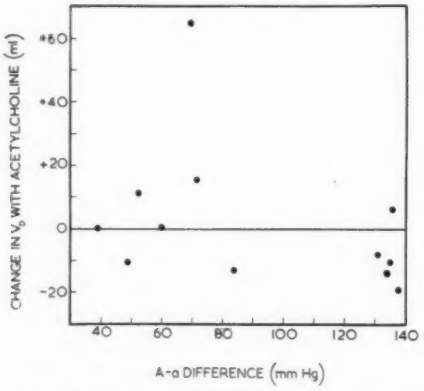

FIG. 6. CHANGE IN PHYSIOLOGICAL DEAD SPACE DURING THE INFUSION OF ACETYLCHOLINE PLOTTED AGAINST THE A-a DIFFERENCE MEASURED BEFORE THE INFUSION. All measurements taken at rest, breathing 47 per cent oxygen.

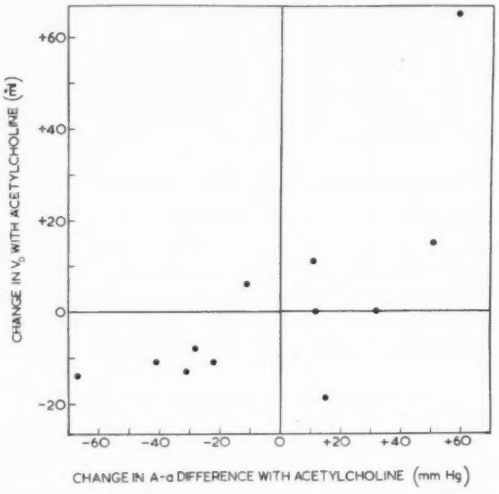

FIG. 7. CHANGE IN PHYSIOLOGICAL DEAD SPACE PLOTTED AGAINST THE CHANGE IN A-a DIFFERENCE DURING THE INFUSION OF ACETYLCHOLINE. All measurements taken at rest, breathing 47 per cent oxygen.

II. Intravascular pressures, cardiac output and pulmonary ventilation

Effects of breathing 47 per cent oxygen. The mean cardiac output, oxygen uptake and arteriovenous (A-V) oxygen difference were not significantly different when 47 per cent oxygen was breathed either at rest or during exercise. The mean pulmonary arterial pressure was similar in the two conditions at rest, but during exercise it

was significantly lower when 47 per cent oxygen was breathed ($0.02 < p < 0.05$). Pulmonary wedge pressure, obtained in seven patients only, was unaffected by breathing oxygen either at rest or on exercise, and the observed decrease in the difference between the pulmonary arterial and the pulmonary wedge pressure (ΔP) was not significant. The mean brachial arterial pressure was unaffected. The heart rate at rest was unchanged but during exercise it was less when breathing oxygen ($p < 0.01$). The pulmonary ventilation in the two conditions was similar at rest, but during exercise there was a significant reduction when oxygen was breathed ($p < 0.01$). This reduction resulted from a decrease in both respiratory rate and tidal volume, but only in the latter was the difference significant ($0.02 < p < 0.05$). The respiratory exchange ratio was insignificantly reduced during oxygen breathing.

Effects of acetylcholine, breathing 47 per cent oxygen. The cardiac output, oxygen uptake and A-V difference were unaffected by the infusion of acetylcholine, either at rest or during exercise. The mean pulmonary arterial pressure was less during the infusion of acetylcholine both at rest and during exercise. The mean pulmonary wedge pressure in the seven patients in whom it was obtained increased slightly with acetylcholine both at rest and during exercise. In neither case was the difference significant, nor was the resultant small decrease in ΔP. The mean brachial arterial pressure and heart rate were not significantly altered by acetylcholine, either at rest or during exercise. Pulmonary ventilation, tidal volume and respiratory rate were unaltered, but the respiratory exchange ratio was less during the infusion of acetylcholine, although the difference was not significant.

DISCUSSION

The average value of the A-a difference in these patients at rest is more than twice the average normal value. Of the factors contributing to the total A-a difference, that due to the "membrane component" can be dismissed. Even though the patients probably had a diminished diffusing capacity for oxygen, no appreciable end-capillary gradient would exist at the alveolar oxygen tensions used in the present studies. The total A-a difference therefore consisted of a component due to anatomical right to left shunting, and a component due to the inequality of ventilation-perfusion ratios in individual alveoli.

On the basis of previously published measurements in normal subjects, Farhi and Rahn (10) suggested that, at alveolar oxygen tensions such as were used in these studies, all but 1 to 2 mm Hg of the total A-a difference was due to anatomical shunting. The increased value in the patients with mitral stenosis could therefore be due to increased anatomical shunting or to the reduction in mixed venous oxygen content consequent on the reduced cardiac output. If this were the case, the anatomical shunt would represent an average of 5.1 per cent of the cardiac output, which is about twice the normal figure.

There is, however, evidence from the present studies and from other considerations that anatomical shunts do not contribute such a high proportion to the total A-a difference as appears to be the case in normal subjects. The normal anatomical venous admixture presumably consists chiefly of the drainage of bronchial venous blood into the pulmonary veins and of thebesian venous blood into the left heart. Administered into the pulmonary circulation, acetylcholine is destroyed so rapidly as not to have any effect on the systemic blood vessels, so that blood flow through bronchial and thebesian shunts would not be expected to change. There may in addition be transpulmonary shunts, but histological examination of the pulmonary vessels in patients with mitral stenosis reveals no evidence of the type of transpulmonary shunts seen in some patients with pulmonary hypertension and congenital heart disease (11). Furthermore, in order to explain the observed changes in A-a difference on the basis of altered anatomical shunting, it would be necessary to postulate a qualitatively different effect of the drug on the vessels concerned in different patients with mitral stenosis, which seems improbable.

It is hardly possible, therefore, to explain the observed changes in A-a difference solely on the basis of an alteration of the fraction of the cardiac output flowing through anatomical right to left shunts. It was observed that changes of A-a difference in either direction occurred during both exercise and the infusion of acetylcholine at rest and during exercise. Furthermore, the changes were accompanied by changes in physiological dead

space which were significantly correlated in each situation. This suggests that an important fraction of the total A-a difference was due to unequal ratios of alveolar ventilation to pulmonary capillary blood flow ($\dot{V}_A/\dot{Q}_c$).

The possibility that acetylcholine produced the observed effects by action on the airways with resulting changes in the distribution of ventilation must be considered. There is no published information on this point but it seems unlikely to occur in view of the destruction of the infused acetylcholine before it reaches the systemic circulation. It will also be noted that there was no observed change in total pulmonary ventilation or respiratory frequency, neither was there any alteration in the amplitude of the respiratory variation in pulmonary wedge pressure during the infusion of acetylcholine.

Söderholm and Werkö (4) suggested that acetylcholine lowered the arterial oxygen saturation by dilating preferentially those arteries which supplied underventilated areas of the lung. Even in a normal person some variation occurs in the degree of ventilation of individual alveoli. In patients with mitral stenosis this variation might conceivably be more than in normal people, although there does not appear to be any direct information on this in the literature. The effect of local underventilation of alveoli is to lower the oxygen tension in those alveoli. There is evidence that a local reduction in oxygen tension causes a local reduction in the blood flow (12, 13). Operating to perfection, this mechanism would result in the maintenance of equal $\dot{V}_A/\dot{Q}_c$ ratios throughout the lungs even though ventilation were unevenly distributed. The infusion of acetylcholine appears to dilate preferentially pulmonary arteries which have been constricted by hypoxia (14). Thus the effect of acetylcholine would be to increase the flow of blood through the hypoxic areas of the lung and simultaneously direct the flow of blood away from the well ventilated areas. If the patient were breathing air, this would cause a decrease in the arterial oxygen saturation, as suggested by Söderholm and Werkö. If the "distribution component" of the venous admixture contributed an important part to the A-a difference at the oxygen tensions existing in the present study, then this action of acetylcholine would be expected to increase the A-a difference and increase the physiological dead space. In those patients with a mean control pulmonary arterial pressure of less than 40 mm Hg, this is in fact what happened, and the above explanation appears plausible. It ignores the fact that it seems unlikely that any portion of the lung could be, strictly speaking, hypoxic while breathing 45 per cent oxygen. Nevertheless, the response of the pulmonary vessels to varying levels of alveolar oxygen tension may well be a graded one so that a relatively hypoxic section of the lung would still receive proportionately less blood.

The diminution in the A-a difference and in the dead space, which was caused by acetylcholine in patients with higher pulmonary arterial pressures, cannot be due to the same mechanism. It is, however, possible to explain this effect on the basis of a primary abnormality in the distribution of the flow of blood to various parts of the lung. Histologically, the smaller pulmonary arteries are not uniformly abnormal, neighboring vessels showing differing degrees of structural change. It is known that the structural abnormalities of the muscular pulmonary arteries which occur in mitral stenosis are most evident in the lower lobes (15). Angiograms also show the pulmonary arterial branches to be most affected at the lung bases (16). There is, therefore, strong evidence of a nonhomogeneity in the supply of blood to the lungs in patients with mitral stenosis and it would be reasonable to assume that this nonhomogeneity increases with increasingly severe pulmonary vascular disease. The presence of such a maldistribution of blood flow would, in the presence of a uniform or randomly uneven distribution of ventilation, give rise to an inequality of ventilation-perfusion ratios. Hence the A-a difference and the physiological dead space would be increased, and this is consistent with the observed correlation between pulmonary arterial pressure and physiological dead space. The lack of correlation between pulmonary arterial pressure and A-a difference may be the result of the variable magnitude of that portion due to anatomical shunting, which would tend to mask any relationship that might exist between pulmonary arterial pressure and the "distribution component." The structurally abnormal vessels may no longer respond to the stimulus of local variations in alveolar oxygen tensions, and this would further widen the distribution of $\dot{V}_A/\dot{Q}_c$

ratios. It might be supposed that the dilating effect of acetylcholine would be strongest in those vessels in which the media had become most hypertrophied. In this case the action of acetylcholine would be to increase the flow of blood through areas with a high ventilation-perfusion ratio and to decrease the blood flow through areas with a low ventilation-perfusion ratio. Thus the effect would be to diminish the physiological dead space and to diminish the A-a difference.

The reason for the different action of acetylcholine at two different stages of the disease may therefore be seen in two overlapping processes. The first is the uneven distribution of ventilation, present to some extent at all stages. The local compensatory vasoconstriction which occurs in the underventilated areas is released by acetylcholine which therefore causes an increased A-a difference and physiological dead space. As the disease progresses, a primary maldistribution of the blood supply becomes increasingly important. Here the main action of acetylcholine is again on the more constricted vessels. In these circumstances, however, the increase in local blood supply occurs in parts of the lung irrespective of their local ventilation and alveolar oxygen tension, so that the effect is to diminish the A-a difference and dead space. With an increasing pulmonary arterial pressure the influence of a maldistribution of blood comes to predominate over the influence of a maldistribution of air, the change occurring with a mean pulmonary arterial pressure of about 40 mm Hg. Although it might appear that the present findings conflict with those of Söderholm and Werkö, it will be noted that in only one of the patients studied by these authors did the pulmonary arterial pressure exceed 45 mm Hg at rest. Their results are not therefore inconsistent with the above hypothesis.

The hemodynamic effects of breathing 47 per cent oxygen at rest were not marked. The cardiac output was not significantly changed, and the pulmonary arterial pressure was reduced by an insignificant amount. The changes during exercise, however, were more definite and, although cardiac output was not altered, pulmonary arterial pressure and heart rate were significantly reduced. The data are not sufficiently complete to state the mechanism of the reduced pulmonary arterial pressure with certainty, although the unchanged cardiac output with a reduction of the pressure drop across the lung suggests that oxygen acted as a vasodilator. The reduction in pulmonary ventilation during exercise is of similar degree to that observed by Cotes (17) in patients with mitral stenosis breathing 100 per cent oxygen. The infusion of acetylcholine during 47 per cent oxygen breathing did not appreciably alter cardiac output or pulmonary ventilation either at rest or during exercise. Pulmonary arterial pressure was reduced both at rest and during exercise, as were also the corresponding values for ΔP, although none of these changes was significant. These findings are qualitatively similar to those of Bateman, Davidson, Donald and Harris (18) for patients breathing 100 per cent oxygen.

SUMMARY

The infusion of acetylcholine in patients with mitral stenosis breathing 47 per cent oxygen produced variable changes in difference between alveolar and arterial oxygen tension (A-a difference) and physiological dead space both at rest and during exercise.

In general, A-a difference and physiological dead space either increased or decreased together. An increase was seen principally in patients with lower resting pulmonary arterial pressures, while in patients with higher pressures both quantities tended to decrease.

A hypothesis is suggested to explain these observations according to which a progressive unevenness of distribution of blood flow to the lungs develops in mitral stenosis. This finally predominates over the uneven distribution of ventilation, the adjustments to which probably account for the observed changes in the patients with less severe disease.

It was incidentally observed that breathing 47 per cent oxygen lowers the pulmonary arterial pressure, heart rate and pulmonary ventilation during exercise.

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GAMMA GLOBULIN COMPLEXES IN RHEUMATOID ARTHRITIS AND CERTAIN OTHER CONDITIONS

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Recent studies have demonstrated that the factors responsible for the various serological reactions currently employed for the diagnosis of rheumatoid arthritis represent macroglobulins which, in the isolated state, have a corrected sedimentation coefficient of approximately 19S (1–5). In serum and plasma, these proteins exist as complexes with low molecular weight γ-globulins and show a higher sedimentation rate, approximately 22S (6, 7). It has become clear that the rheumatoid factors have a specific affinity for ordinary γ-globulin showing a behavior, particularly in certain of the serological tests, like that of anti-γ-globulin antibodies. The possibility has been raised that the rheumatoid factor complex in serum represents an antigen-antibody complex.

During the course of investigation of the rheumatoid factors and their complexes through ultracentrifugal analyses of sera showing high titer reactions in the latex fixation test and γ-globulin precipitin test, another group of γ-globulin complexes was encountered. These had sedimentation rates between 9 and 17S and were dissociated in urea and acid buffers in a manner similar to the previously observed dissociation of the rheumatoid factor complexes. Again the question arises as to whether these are antigen-antibody complexes or simple aggregates of γ-globulin.

Gamma globulin components in the 9 to 12S range have been observed in many preparations of normal Fr II γ-globulin. The presumption has been that these represent aggregates formed in the process of chemical fractionation; they were not found in γ-globulin isolated by electrophoretic convection (8). Certain myeloma proteins have been found in which either the major component or accessory components have sedimentation rates in this range (9, 10). This has been particularly true of some of the more rapidly migrating myeloma proteins (11). Very little work has been carried out on these components particularly from the standpoint of association and dissociation, although Pedersen (12) described a myeloma protein with an s-rate of approximately 11S at high protein concentrations and approximately 7S at lower concentrations. Similar abnormal proteins have been encountered in certain ill-defined disorders (13). Particularly pertinent is a recent report of a macroglobulin studied by Rees and Resner (14) that could be dissociated in acid. Certain sera with similar proteins probably have been considered to represent macroglobulins of the Waldenström type and have been reported as such in the literature (15, 16), although the sedimentation rate of the main abnormal protein was lower than the 18 to 20S range of the proteins described by Waldenström. Macroglobulins of the latter class are a homogeneous group and are known to be stable proteins which do not dissociate in urea and acid and alkaline buffers (17). They have specific immunological properties and dissociate in the presence of agents breaking disulfide bonds (18).

In the present study, sera from 11 different patients, 7 with classical rheumatoid arthritis, were found to contain multiple γ-globulin complexes. These were readily detected in whole serum and plasma by direct ultracentrifugal analysis. They did not possess rheumatoid factor activity but were associated in the same sera with large amounts of rheumatoid factor. Investigation of the association and dissociation reactions of these complexes was carried out and certain of their properties defined. Evidence for and against the hypothesis that they represent antigen-antibody complexes of the γ-globulin-anti-γ-globulin type was sought.

MATERIAL AND METHODS

Six of the 11 patients whose sera were specifically analyzed in this study were observed in the clinic and outpatient department of the Rockefeller Hospital. Three of the remainder represented patients in various New York

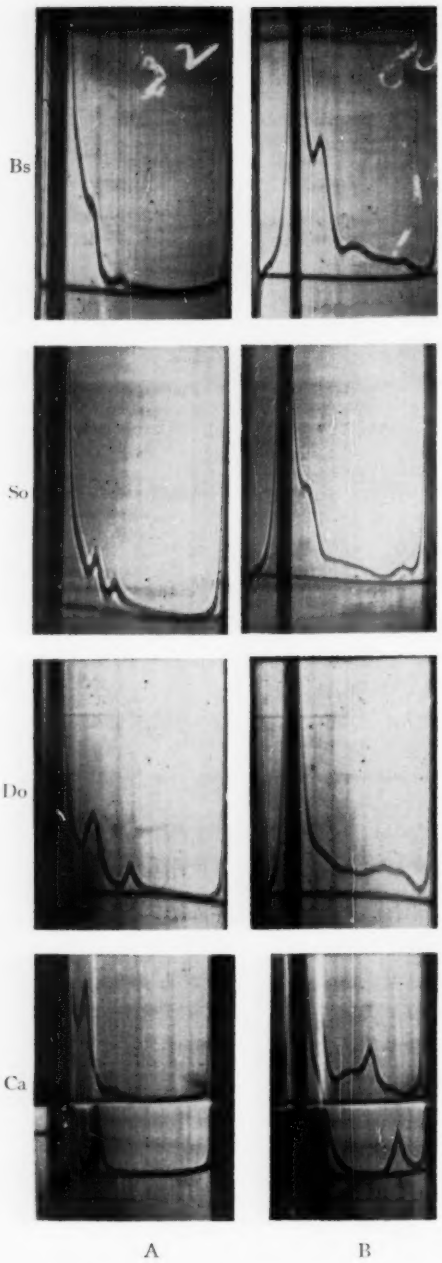


FIG. 1. ULTRACENTRIFUGAL PATTERNS FROM FOUR SERA SHOWING THE INTERMEDIATE TYPE COMPLEXES. Photograph A was taken at 32 minutes and B at 80 minutes, with all sera diluted with an equal volume of isotonic saline. The bottom patterns are from a control serum showing primarily the normal 19S peak.

TABLE I

Clinical diagnosis and age of the 11 patients included in the study

Patient	Diagnosis	Age
Do	Rheumatoid arthritis	28
So	Rheumatoid arthritis	19
Cr	Rheumatoid arthritis	38
Ro	Rheumatoid arthritis	44
An	Rheumatoid arthritis	41
Wa	Rheumatoid arthritis	46
St	Rheumatoid arthritis	58
Bs	Hepatosplenomegaly, slight arthritis	66
Fi	Idiopathic hepatomegaly and purpura	55
Co	Idiopathic purpura	24
Ca	Fever, purpura, lymphadenopathy; unknown etiology	14

hospitals whose sera were sent for ultracentrifugal analysis either for rheumatoid factor complex or for the usual macroglobulinemia. All of these patients were secondarily seen by one of the authors who attempted to evaluate the diagnosis of the local physician. One serum (Ro) came from a patient with rheumatoid arthritis under the care of Dr. Evan Calkins in Boston. Another, (Co) came from Dr. Corral in Havana, Cuba; this patient was not seen by the authors but he did not have arthritis. The clinical diagnosis was idiopathic purpura. The criteria for including the 11 cases in this study were that their sera contained more than 10 per cent of the total protein in the form of aggregates in the 9 to 17S range (called intermediate complexes) and that these aggregates dissociated in acid buffers. Table I shows the age and clinical diagnosis of the 11 patients.

The ultracentrifugal and electrophoretic techniques were similar to those described previously (19). All specifically designated s-rates were obtained by infinite dilution extrapolation from sedimentation constants obtained at 2 or more protein concentrations. In some experiments a highly purified 19S Waldenström macroglobulin was added to serum or serum fractions to verify the position of the 19S peak where this was obscure. The position of the 19S components in Figure 1 was obtained in this way. The use of standard preparations of known s-rate for delineating proteins in complex mixtures was illustrated in a previous study (5). Euglobulin preparations were routinely made by diluting serum with 10 vol of de-ionized distilled water. In certain instances, mentioned below, dialysis against phosphate buffer, pH 6.0, $\Gamma/2$ 0.02, was utilized to prepare euglobulin components. Spontaneous precipitates were determined by the saline dilution method of Christian (7). Chromatographic experiments were carried out by the procedure of Sober, Gutter, Wyckoff and Peterson (20), utilizing a continuous pH and ionic strength gradient.

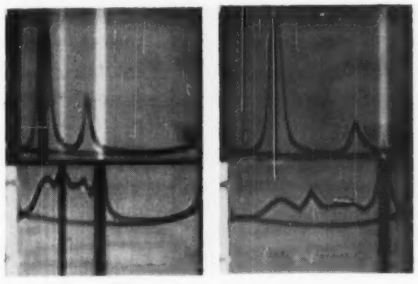
The serological tests were carried out primarily as described previously (6). The latex fixation was determined after overnight standing in the cold room. A 1/20 basic agglutination titer was used in the sensitized sheep cell test. The human Rh sensitized cell test was a modification of the technique of Waller and Vaughan (21) utilizing a single Rh antiserum for all determinations. This was selected from a group of 30 Rh antisera for its reactivity with different rheumatoid factors. Inhibition reactions were studied by a modification of the procedure of Grubb and Laurell (22). The method was standardized with known inhibitor and non-inhibitor sera kindly provided by Drs. Laurell and Grubb.

RESULTS

Whole serum analyses. The sera of 11 different patients were found to contain a wide assortment of abnormal components visible by direct ultracentrifugal analysis of whole serum. Figure 1 illustrates the patterns from four of these cases along with a control serum showing slight elevation of the normal 19S component. In normal and control sera very little material is visible between the 19S and 7S components, and the pattern comes close to the baseline in this area. However, in each of the pathological sera illustrated, a continuous spectrum of components extends through this area. This is most apparent in the B frames which were taken at a later time. In addition, the first three sera llustrated—Bs, So and Do—showed a more rapidly sedimenting component which is visible only in the A frames and has completely sedimented in the B frames. It has a corrected s-rate between 22 and 28S in these sera and represents in serum So and Bs the "22S" component previously described as characteristic of certain rheumatoid arthritis sera (6). In serum Do it had a considerably higher s-rate, approximately 28S. Serum Ca does not show such a component as a well defined peak, although some rapidly sedimenting material is visible.

Efforts to calculate the exact s-rates of the intermediate class substances were unsatisfactory because of the extreme heterogeneity of the components and because some differences were encountered at different dilutions of serum. Most of the sera showed some accentuation of the general elevation in the 10 to 12S range and also in the 14 to 16S range, but this was not always apparent, particularly in very late frames where the marked diversity of components was most apparent. Serum Ca showed somewhat more of 14 to 16S material, as shown in the B frames of Figure 1, sedimenting rather more slowly than the control 19S. Here again, however, later frames showed a complete spectrum of components from 7 to 19 S. The concentration of the intermediate components showed considerable variation in the different sera. As mentioned above, the minimal concentration set for inclusion was 10 per cent of the total protein. The highest concentration was found in serum St,[1] where approximately 43 per cent of the total protein was in the form of intermediate complexes. Sera Ca, Fi and Do had 31, 35 and 39 per cent, respectively. The remainder ranged from 10 to 30 per cent.

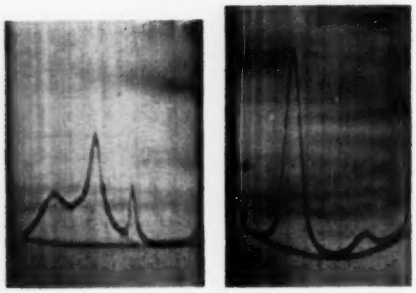
Euglobulin and γ-globulin fractions. A characteristic property of the 11 sera showing the intermediate components by ultracentrifugal analysis was a positive Sia test and a considerable precipitate on simple dilution with distilled water. In several instances this finding led to the initial ultracentrifugal analyses because of the possibility of macroglobulinemia of the Waldenström type. Analyses of these precipitates following solution in 5 per cent NaCl showed the characteristic intermediate components noted in whole serum. The lower portion of Figure 2 illustrates the pattern of the euglobulin fraction of serum Do at early and later times. The major peak is a rapidly sedimenting component with $s^{\circ}_{20,w} = 22.8S$, but



A B

FIG. 2. ULTRACENTRIFUGAL PATTERNS OF EUGLOBULIN FRACTIONS FROM SERUM DO AT EARLY AND LATER TIMES UTILIZING DOUBLE CENTRIFUGE CELLS. Lower patterns show the euglobulin in 5 per cent NaCl solution. The upper patterns are the same euglobulin concentration in glycine-HCl buffer at pH 3.5. There is complete disappearance of the intermediate complexes in the acid buffer.

[1] Dr. Lospalluto and Dr. Ziff have also observed intermediate type complexes in this serum as well as in certain other rheumatoid arthritis sera (23).



A B

FIG. 3. PATTERNS OF PURIFIED EUGLOBULIN FRACTION FROM SERUM Do (A) IN 5 PER CENT NaCl, AND (B) IN 6 M UREA. Complete dissociation of the intermediate complexes occurred in the urea.

the 9 to 17S intermediate components are also very prominent. Figure 3A illustrates another euglobulin pattern from this same serum. In this case the intermediate components were concentrated by differential euglobulin precipitation at pH 8.3. The higher s-rate components (19 to 28S) tended to precipitate more rapidly at lower pH. It became evident that both the heavy component and the intermediate components were the fractions causing the high euglobulin levels. Serum Ca differed from the others in that the euglobulin precipitate did not dissolve in 5 per cent NaCl or dissolved only slightly. In this case the abnormal components which are present in whole serum appeared to be very readily altered so that they could not be redissolved in various buffers even in the presence of added albumin. However, in glycine-HCl buffer at pH 3.5 or in 6 M urea, soluble prepartions were readily obtained. These then consisted of almost pure 7S material with a small 19S peak.

Zone electrophoretic experiments in various supporting media indicated that both the rapidly sedimenting component and the intermediate peaks were γ-globulins. Such analyses were carried out on sera Do, Ca and Bs. A considerable portion of the elevated γ-globulin noted in these sera was accounted for by the abnormal components. In serum Do the intermediate complexes made up 70 per cent of the γ-globulin. Some problems of recovery of these components were encountered following electrophoresis in these experiments, but they were not found in electrophoretic fractions other than the γ-globulin.

Dissociation. The intermediate peaks described above were readily and completely dissociable in the presence of 6 M urea or glycine-HCl buffer, pH 3 to 3.5, giving rise to ultracentrifugally homogeneous components of the 7S class. The 22S peaks and those with higher s-rates were dissociated to a mixture of 7S and 19S fractions. Typical results of this type are shown in Figures 2 and 3. The euglobulin fractions which prior to dissociation consisted of many components, showed only two peaks, 7 and 19S, after acid or urea treatment. These conditions are known not to dissociate the 19S macroglobulins. Acetate and phosphate buffers in a pH range as high as 5.5 produced partial dissociation as did 4 M urea. Table II shows the results of a typical experiment with a euglobulin preparation from serum Bs. The intermediate components with s-rates of 10.5, 12.6 and 15.9 disappeared completely with a tremendous increase in the area of the 6.5S peak following dissociation in acid. Similar dissociation results were also obtained with whole serum with marked reduction of the intermediate components (Figure 4). The results here were clear-cut when large amounts of complexes were present as in the two sera illustrated. However, when small amounts of complexes existed, difficulties were encountered due to interference by certain serum proteins and production of minor components resembling the complexes in normal serum with certain of the acid buffers. Gamma globulin preparations from sera Do, Bs and Fi showed complete dissociation in the acid buffers. Mercaptoethanol, which is known to dissociate the Waldenström type macroglobulin (18) had little or no effect on the intermediate peaks from euglobulin or γ-globulin preparations.

TABLE II

Corrected sedimentation rates and areas of euglobulin components from serum Bs before and after dissociation in acetate buffer

NaCl, 0.2%		Acetate buffer pH 4.2, Γ/2 0.1	
$s^{\circ}_{20,w}$	% of total	$s^{\circ}_{20,w}$	% of total
6.6	22	6.5	82
10.5	26		
12.6	11		
15.9	14		
18.8	10	18.5	18
21.6	17		

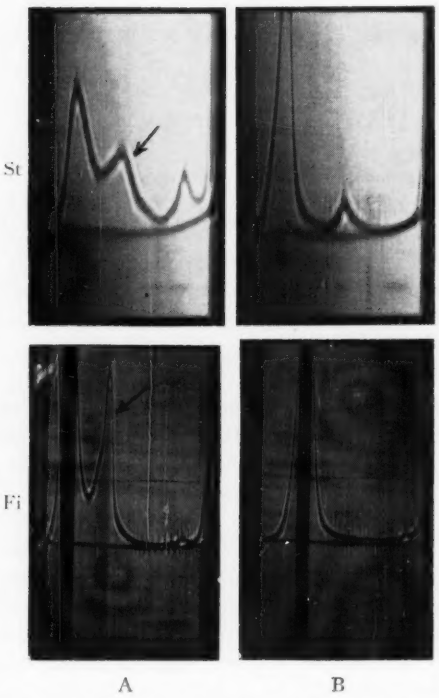


FIG. 4. PATTERNS OF THE WHOLE SERUM OF PATIENTS ST AND FI DILUTED IN ISOTONIC SALINE (A) AND ACETATE BUFFER, pH 4.5 (B). The arrows indicate the large intermediate complexes which disappear in the acetate buffer.

Following dissociation, removal of the urea or acid by dialysis resulted in reformation of many intermediate complexes, although the exact pattern observed in the original euglobulin fraction or serum was not quite reproduced. Figure 5 illustrates the distribution of components following dissociation in urea and again after dialysis against barbital buffer, pH 7.8. The original euglobulin fraction used was from serum Do and resembles that shown in Figure 2B, although this preparation contained considerably less of the large 22S component shown in Figure 2B. The intermediate complexes were reformed in this experiment, but those with the s-rates above 19 were not.

In view of the complete dissociation of the intermediate complexes to 7S units, the possibility arose that the components might be antigen-antibody complexes of the type γ-globulin-anti-γ-globulin. It seemed of interest to see the effect of adding additional γ-globulin. Several of the sera showing the largest amount of intermediate components contained very little free γ-globulin in the serum. This was particularly true of serum Do in which only a small 7S component was visible in the ultracentrifugal pattern of whole serum and the γ-globulin fraction isolated by zone electrophoresis. The addition of Fr II γ-globulin (freed of aggregates by preparative ultracentrifugation) showed a striking effect. The whole range of complexes was markedly affected. The larger components disappeared almost entirely and resulted in the accumulation of a single large peak with an s-rate of approximately 10S. Each of the three sera shown in Figure 1 demonstrated a similar result both for the whole serum and for the euglobulin fraction. Figure 6 illustrates this effect for serum Ca. An amount of albumin equal to the γ-globulin was added as a control and no change was noted. The complexes in the presence of γ-globulin showed a marked shift to smaller complexes. Preparations of euglobulin Do, similar to that shown in Figure 2, illustrated this effect of added γ-globulin very dramatically.

As was mentioned above, certain of these sera contained complexes with an s-rate which was higher than the previously determined 22S for the usual rheumatoid factor complex. These ranged from 28 to 31S. This was particularly true of serum Do which showed extremely high titers in the rheumatoid factor tests. No 22S component, as is usually seen in such patients, was observed, and the accumulated evidence indicated that the rheumatoid factor in this serum was in the 28S component (Figure 1). This component disap-

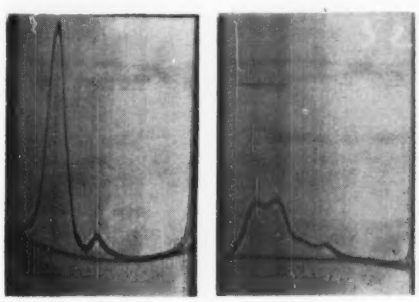


FIG. 5. REFORMATION OF INTERMEDIATE COMPLEXES FROM A DISSOCIATED EUGLOBULIN FRACTION. A, euglobulin in urea; B, euglobulin in urea followed by dialysis against barbital buffer.

peared after precipitation with aggregated γ-globulin. Euglobulin fractions on the other hand showed a large 22S component. This is visible in the lower portion of Figure 2, sedimenting slightly faster than the 19S component visible in the upper frames after dissociation in acid buffer. At least five examinations of whole serum Do at different dilutions always showed the rapidly sedimenting peak with an s-rate higher than the usual 21 to 23S for the rheumatoid factor. However, on the addition of 7S γ-globulin to the whole serum, as described in the preceding paragraph, this rapidly sedimenting peak showed a shift in s-rate to close to 22S with a definite loss of area. It appeared that in the presence of low 7S γ-globulin levels in this serum, the rheumatoid factor complexed with intermediate type aggregates of γ-globulin giving the higher s-rates. When excess 7S γ-globulin was added, these aggregates were replaced by the usual 7S moieties and the rheumatoid factor complex assumed its usual s-rate. Just why the euglobulin fraction did not show the 28S component was not entirely clear; relatively more 7S γ-globulin appeared to be present than in whole serum, which may have arisen from dissociation of some of the complexes, and this may have caused a shift to the lower s-rate. The 28S complex was present, however, in the γ-globulin isolated by zone electrophoresis.

Serum St, which had the greatest amount of intermediate complexes, also showed a large, rapidly sedimenting peak. In this case the s-rate was calculated as $s^{\circ}_{20,w} = 31S$. This peak also showed a marked shift in the presence of excess 7S γ-globulin with conversion to the usual 22S class.

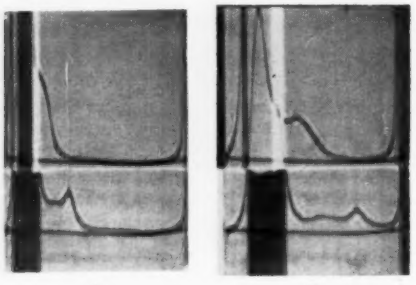


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FIG. 6. THE EFFECT OF ADDITION OF ISOLATED 7S γ-GLOBULIN ON THE DISTRIBUTION OF THE INTERMEDIATE COMPLEXES. Lower patterns with albumin added show no effects; upper patterns, with γ-globulin added, show a shift to smaller complexes.

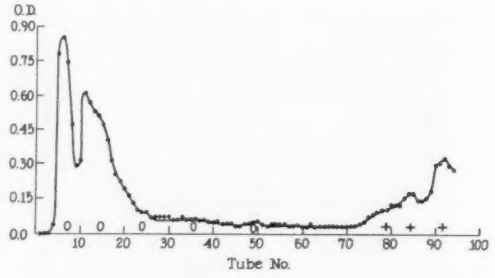

FIG. 7. DISTRIBUTION OF COMPONENTS OBTAINED BY DEAE COLUMN CHROMATOGRAPHY OF THE EUGLOBULIN FRACTION OF SERUM Do. The signs under the peaks designate rheumatoid factor activity in the sensitized Rh_0 test. The intermediate complexes were found in the peak of tubes 11 to 20 and were devoid of activity. The 19S class fractions appeared beyond tube 70 and showed the activity.

Complex formation with addition of γ-globulin. One serum (Fi) demonstrated a marked increase in intermediate complexes when γ-globulin was added, an effect that was not observed with the other sera where simply a shift to smaller complexes occurred. Here there was evidence that free material was present in the serum which was capable of reacting with added γ-globulin. The addition of 7S γ-globulin to this serum actually produced a fall in the 7S peak of the serum with a marked increase in material sedimenting at approximately 10S.

Studies with purified complexes. Purified preparations of intermediate complexes, such as those illustrated in Figure 3A which had been prepared by differential euglobulin precipitation, still contained substantial amounts of 7S and 19S γ-globulin. Further purification was achieved by zone ultracentrifugation in a sucrose density gradient. Approximately 25 fractions were taken from the bottom of the tube by means of a fraction collector. Protein analyses on these fractions showed three major peaks corresponding to those shown in Figure 3A. The middle peak, when examined in the analytical ultracentrifuge, showed 70 per cent 9 to 14S material and 30 per cent 7S. Further efforts at greater purification of this fraction, by both differential precipitation and density gradient centrifugation, resulted in increasing quantities of 7S γ-globulin and small complexes in the 9 to 10S range. It became apparent that the complexes

continually dissociated with purification and that they were extremely difficult if not impossible to isolate completely. This behavior was very similar to that previously noted for the 22S rheumatoid factor complex; with purification, dissociation occurred and 19S rheumatoid factor resulted.

Considerable purification was also achieved on diethylaminoethyl (DEAE) cellulose columns and the complexes caused the appearance of a large peak not ordinarily observed in the separation of serum. The pattern obtained for the euglobulin from Do is illustrated in Figure 7. The complexes were found in the peak of tubes 11 to 20. However, as in the case of the purified fractions isolated above, considerable 7S material was observed by ultracentrifugal analysis of pooled material from these fractions.

Both in the crude and in the purified state the complexes dissociated in acid buffers to units which were indistinguishable from ordinary 7S γ-globulin. Mixing experiments with added 7S gave single peaks. Immunological analyses were also attempted with the middle fraction isolated from the density gradient tube as described above. Both by Ouchterlony plate analysis and immunoelectrophoresis, no antigen other than ordinary 7S γ-globulin was detected. In these studies a number of antisera was employed, made against whole serum, Fr II and whole electrophoretic γ-globulin. Antisera were not made against the complexes. Efforts to detect materials with β_{2A} or β_{2M} specificity in the complexes were unsuccessful.

One purified preparation was obtained from serum Fi by zone electrophoresis of a euglobulin fraction. This consisted of 81 per cent 10S material and the remainder mainly of 7S. This preparation contained no visible 19S peak. Carbohydrate assays were performed following a second electrophoresis in polyvinyl chloride medium as described previously (24). Each fraction contained approximately 1.5 per cent hexose sugars. There was no evidence of a carbohydrate-rich material in the complexes, and the values were very similar to those obtained for ordinary 7S γ-globulin (24).

Incidence and distribution of complexes. It is difficult from this study to determine the exact incidence of the intermediate type complexes either in rheumatoid arthritis or in other disease conditions. The 11 cases described above were selected because of extremely large amounts of the intermediate complexes at a time when wide varieties of sera were examined in the ultracentrifuge. These were from patients with elevated γ-globulin levels and particularly from those showing high euglobulin fractions and positive Sia tests. Patients with liver disease, macroglobulinemia, myeloma, leukemia, parasitic disease, sarcoid, and others, were studied. Also, a large number of high titer rheumatoid arthritis sera was examined. Eight of the 11 cases came from the latter group. Seven were typical severe rheumatoid arthritis patients with two showing a positive lupus erythematosus test along with severe arthritis. The eighth case, Bs, although showing some rheumatoid arthritic changes, was atypical and represented somewhat of a diagnostic problem because of liver enlargement. All eight of these sera were characterized by extremely high titers in the various tests for rheumatoid factor. Of the remaining three sera, one (serum Ca) was from a 14 year old girl who had no evidence of arthritis and suffered from intermittent low-grade fever, purpura and general glandular enlargement of unknown etiology. Lymph node biopsy was read by one pathologist as lymphoblastoma, by another as nonspecific lymphadenitis. The illness was present without progression for four years. This serum

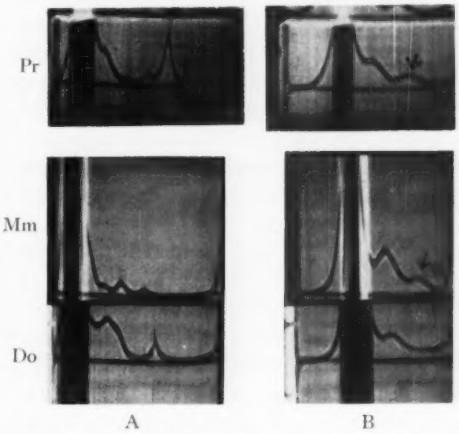

FIG. 8. ULTRACENTRIFUGAL PATTERNS OF TWO ADDITIONAL RHEUMATOID ARTHRITIS SERA, SHOWING SMALLER AMOUNTS OF INTERMEDIATE COMPLEXES. Serum Do is illustrated again for comparison. The arrows in the later (B) frames show material of approximately 10S in each case.

TABLE III

Results of rheumatoid factor tests in the 11 patients showing intermediate complexes

Patient	Latex fixation	Sensitized sheep cell	Tanned cell
Do	640,000	80,000	
So	160,000	20,000	
Cr	20,000		
Ro	10,000	1,280	
An	5,000	5,000	
Wa		2,560	
St	800,000	200,000	
Bs	20,000	640	10,000
Fi	20,000	16	
Co	2,560	128	
Ca	10,000	2,560	10,000

differed somewhat from the others in a number of respects. First, the titers in the various serological tests were not as high as for most of the other cases. Second, a marked precipitate formed on simple dilution of the serum with saline even at room temperature. This interfered with the precipitin reaction with aggregated γ-globulin and perhaps with the other serological tests. Analysis of the precipitate produced with aggregated γ-globulin after solution in acid buffers showed primarily 7S γ-globulin and not the usual predominance of the 19S fraction. Third, the insolubility of the euglobulin fraction in salt solutions, mentioned above, stood in marked contrast to the euglobulin of the other sera studied. The second nonrheumatoid serum was from a young woman with very severe purpura which was diagnosed as hyperglobulinemic purpura. She has been extensively studied by Dr. Corral of Havana, Cuba, and her case will be published in detail separately. The third patient of this group without arthritis (Fi) showed relatively few clinical symptoms. Enlargement of the liver and spleen led to hospitalization and the finding of the protein abnormalities. This patient also had one episode of generalized purpura.

A high incidence of intermediate type complexes in rheumatoid arthritis, relative to other conditions, appears likely from the distribution of the cases described above. In addition to the seven or possibly eight cases described, a considerably larger group of rheumatoid arthritis sera appeared to show intermediate complexes in smaller amounts. Figure 8 illustrates the patterns from two of these cases—both patients with severe rheumatoid arthritis and uniquely high titers in the serological reactions. For comparative purposes the pattern of serum Do is shown again. The early frames (A) show 22S components for Pr and Mm, giving an extremely high peak in Pr which sediments just ahead of the 19S peak. In Mm a third peak is visible, sedimenting slightly more slowly than the 19S component, with a calculated $S^{\circ}_{20,w}$ of 16S. The later frames (B) show the delineated 7S component in all three sera in the mid-portion of each frame. In Pr and Mm a clear peak is visible sedimenting ahead of the 7S which has an s-rate of approximately 10S in each case. These peaks resemble those in serum Do and the others described earlier when excess 7S γ-globulin is added to the serum. They are definitely abnormal components and normal serum has little or no material sedimenting in this position. Most other high titer rheumatoid arthritis sera showed similar components and the predominant abnormal component was usually in the 9 to 11S range. These sera have not been included in the present series because of less certainty regarding these components. They were more difficult to study because of their low concentration, and it was not always clear that they dissociated to 7S units. However, it appears likely that they represent smaller amounts of complexes similar to those in the 11 sera described above.

Activity of complexes. Table III shows the titer of the eight sera in various serological tests for rheumatoid factor. Table IV demonstrates the titer of three of the sera in the Rh sensitized cell test. In addition, the inhibition titer of these sera in the Gm inhibitor system is indicated (*22*). The anti-Rh_0 system used in this experiment gives

TABLE IV

Inhibition titers in the Grubb system of three of the sera from patients showing γ-globulin complexes

Patient	Agglutination titer*	Inhibition titer†
Bs	1/160	1/3,096
Do	1/1,600	1/2,048
St		1/4,096
Ca	1/40	1/2,048
Inh (H.F.)	0	1/64
Non-Inh (H.H.)	0	1/1

* The Rh serum used for these titers was separate from that used for inhibition titers.

† All serum inhibition titers measured with standard rheumatoid serum no. 19N (1/20 dilution) for Gm-grouping.

a good distinction between the hereditary γ-globulin groups of Grubb and Laurell (*22*) without showing strong agglutination with most rheumatoid sera. It is clear that the sera under study showed very great inhibitor activity compared with a normal serum belonging in the Gm(a⁺) or inhibitor group. Such inhibitor titers were not encountered with other pathological sera with the exception of certain myeloma sera, and the evidence suggests that the complexes were responsible for the inhibition.

Considerable efforts were directed toward finding additional biological activity for the intermediate components. The accumulated evidence indicated that they did not show typical activity as rheumatoid factors in the various serological tests. Density gradient analysis of these sera by methods described previously (*25*), showed the major activity in the bottom fractions containing 19S or greater classes of proteins. The intermediate fractions which were demonstrated to contain the complexes were devoid of activity. This was also the case with the complexes isolated by chromatography (Figure 7). There was no activity in the peak containing the complexes, and all the activity was in the later fractions containing the 19S class proteins. Most of the isolated complexes also failed to react in the precipitin test with γ-globulin aggregates and soluble antigen-antibody complexes. However, the complexes from sera Ca and St, which were relatively insoluble, showed intensified precipitation in the presence of aggregated γ-globulin, although they failed to give positive reactions in the serological tests. Evidence was also obtained with several sera that the complexes intensified the precipitation reaction with aggregates and caused larger amounts of 7S γ-globulin to appear in acid-dissolved precipitates (*1*). The question of whether this indicated actual participation in the reaction, or simply coprecipitation as a result of their general insolubility, was not entirely clear.

Some evidence was obtained that these complexes played a primary role in spontaneous precipitation from serum (*7*). Certainly, serum Ca gave an insoluble precipitate on standing, and on dilution with isotonic saline a large amount of precipitate formed. Here the rheumatoid factor did not appear to be directly involved, and the precipitate was almost entirely composed of the intermediate aggregates. Sera Bs and St also showed considerable spontaneous precipitate. However, Do which had both large amounts of intermediate complexes as well as extreme titers in the rheumatoid factor tests, showed almost no precipitate in whole serum standing in the cold room and only a small amount on 1/10 dilution with isotonic saline and standing in the cold room. The remaining sera were not specifically checked from this point of view.

DISCUSSION

The ready dissociation of the intermediate type components described above indicates clearly that they represent complexes. The dissociated unit showed a homogeneous peak in the ultracentrifuge with an s-rate of approximately 7S. Certainly, they were in part ordinary 7S γ-globulins as shown by the immunological reaction with antiserum to normal γ-globulin. No evidence for additional constituents was obtained and the accumulated evidence suggested that the complexes were composed entirely of 7S γ-globulin units. The fact that normal 7S γ-globulin, when added to these complexes, had a profound effect on their distribution was of special interest. The larger complexes disappeared and primarily one type with an s-rate of approximately 10S remained. These results suggested that one constituent of the complexes was ordinary γ-globulin which could be influenced and replaced by γ-globulin from other sera. The major question which is raised is whether or not the remainder of the complex is antibody to γ-globulin. Certainly, the effect of extraneous γ-globulin suggests the action of excess antigen on antigen-antibody complexes causing the formation of limiting complexes. In the case of bivalent antibodies, these would be composed of three units of γ-globulin. However, the broadness of the peaks and the spread of the added 7S γ-globulin made it difficult to decide between limiting complexes composed of two or three units of γ-globulin.

Recent evidence has indicated a marked affinity of the rheumatoid factors for the γ-globulin of specific individuals (*26*). The specificity of this reaction offers strong support for the concept that these proteins represent high molecular weight antibodies to γ-globulin. Attempts were made in

the present study to demonstrate a similar specificity of the active portion of the intermediate complexes. Largely for technical reasons this was not accomplished, although further work in this direction is continuing. Some of the effects of added γ-globulin on the complexes could be better explained on the basis of greater reactivity with certain types of γ-globulin than simply on the basis of excess γ-globulin effects. If the active portion of the complexes is antibody to γ-globulin, as is certainly suggested, it must be of a nonprecipitating type in most of the sera studied.

The one serum which showed formation of additional complexes when γ-globulin was added was of special interest in this connection. Here insufficient γ-globulin appeared to be present to combine with the active fraction, and further γ-globulin was required to form the maximal number of complexes. The marked affinity of this patient's γ-globulin for extraneous γ-globulin was clearly evident.

The failure to find complexes of this type larger than 17S in any of the sera suggested a definite limitation on the polymerization and along with their nonprecipitating character raises the possibility of a univalent type of antibody.

The association between the presence of intermediate complexes and rheumatoid factor in the 11 sera described was striking. However, it certainly was not an absolute or direct relationship. All the rheumatoid arthritis sera showing large amounts of the complexes had high titers in the rheumatoid factor tests. On the other hand, a number of other sera with extremely high titers by these serological reactions did not show such large amounts of complexes. Evidence was obtained that many of these did show smaller amounts of similar components but their complete identity with the others was difficult to establish. The association, at least in a number of sera, suggests that the active portion of these complexes represents the 7S counterpart of the 19S group of rheumatoid factors. It is well known in such instances as the typhoid agglutinins and isoagglutinins that 19S antibodies are usually not the sole type but occur along with ordinary 7S antibodies (25). If the 19S rheumatoid factors are indeed antibodies one would expect to find similar 7S types. These would give rise to complexes with ordinary γ-globulin just as the rheumatoid factors give rise to the 22S complex. Several of these sera showed the latter complex along with the intermediate type complexes. It is of special interest that several of the sera showed a large peak with an unusually high s-rate (28 to 31S). Evidence was obtained that this represented rheumatoid factor complexed with intermediate complexes. This component disappeared on the addition of excess ordinary low molecular weight γ-globulin and then became the more usual 22S fraction. It was apparent that a wide assortment of complexes occurred in these sera, so much so that in some instances little or no γ-globulin remained in the uncombined state. The fact that the complexes did not show typical rheumatoid factor activity is not conclusive evidence against their being the 7S counterparts of the 19S rheumatoid factors. It is well known that high molecular weight antibodies are particularly good agglutinators and the 7S antibodies often fail in this respect, as in the case of the Rh antibodies. Furthermore, the combination with other 7S units might block the participation in the agglutination reactions more than in the case of the 19S rheumatoid factors.

No clear results were obtained which suggested a direct harmful biological role for these complexes. It seems possible and indeed almost probable that under certain conditions they would precipitate. This would seem to be particularly likely in the presence of low γ-globulin levels which would tend to favor larger complexes. In most situations excess γ-globulin would keep the complexes small and adequately soluble. It seems somewhat surprising that the intermediate complexes did not precipitate with rheumatoid factor, but this is in line with previous evidence that precipitation occurred only when very large soluble complexes or aggregates were added (26). Perhaps some such precipitation does occur *in vivo*. The problem appears related to that of the spontaneous precipitates described by others as occurring in certain rheumatoid arthritis sera. A final conclusion on this point was not obtained, because certain of the sera with large amounts of complexes did not show significant spontaneous precipitation even after dilution with saline. The complexes also appeared different from the components noted by Christian (7) in certain spontaneous precipitates dissolved in acid. Such conditions would

dissociate the complexes described in this study to ordinary 7S units. Another possible biological activity found for the intermediate type complexes was an inhibition effect in certain of the rheumatoid factor agglutination reactions. This was observed in the Grubb and Laurell Gm(a) system employing sensitized Rh_0 positive human red cells.

Consideration certainly should be given to the possibility that the complexes represent simple aggregates of γ-globulin of the type observed in certain Fr II preparations. Most observers feel that these result from the isolation procedures, but the possibility remains that they represent in part complexes existing in small amounts in normal serum that are concentrated by the isolation procedures. Some γ-globulin preparations isolated from normal serum by zone electrophoresis and normal euglobulin fractions show similar components that appear to be present in the original serum. It seems probable that some of the latter are more closely related to the C'_3 complex recently described (27) which is also a euglobulin. Another possibility is that a γ-globulin is produced, in the group of patients described, which has a specific property of polymerizing with other γ-globulin molecules. The β_{2A}-protein, which is related to ordinary γ-globulin immunologically, has been found to have unusual complexing tendencies, and Heremans has recently described a β_{2A}-albumin complex (28). However, the low carbohydrate content of the complexes and the failure to find significant β_{2A} specificity immunologically, make this possibility unlikely.

It appears highly probable that cases similar to the present group have been noted in the past and classified as Waldenström's macroglobulinemia. The distinction is not readily made unless dissociation experiments are carried out. Both types show positive Sia tests. Serum Fi, illustrated in Figure 4, showed a pattern closely resembling that seen in Waldenström's macroglobulinemia with a huge, relatively sharp peak with an s-rate of approximately 15S. Close inspection of this pattern, however, reveals minor differences even without resorting to dissociation analysis. The main peak has a slight shoulder of lower s-rate. This has never been encountered in the true macroglobulinemias. Also the higher polymers, which are so characteristic of the latter syndrome (17), are missing. The interesting case reported by Conrad, Ramos, Howie and Crosby (29), which was proven by Rees and Resner (14) to have acid-dissociable components in the serum, is called Waldenström's macroglobulinemia. This case was undoubtedly similar to those in the present series, although rheumatoid factor tests were not described. To prevent confusion and since the proteins involved are quite different, it seems better to avoid the term macroglobulinemia in referring to these cases.

Unfortunately, no sensitive method comparable with the serological tests for rheumatoid factors is available to measure the intermediate complexes or their active constituents. It seems probable that if such a procedure were devised a considerably higher incidence in rheumatoid arthritis would be found. Previous experience with the rheumatoid factors has demonstrated the insensitivity of the ultracentrifuge and only the extremely high titer sera showed the 22S rheumatoid factor complex. It is impossible to answer conclusively the question of relationship to clinical manifestations of disease. Certainly, most of the cases showing large amounts of intermediate complexes suffered from severe and long-standing joint disease; but three of the cases showed no arthritis at all. Some of the patients showed idiopathic purpura that might be related to the protein changes but again others in the group failed to show this. Further observations with more sensitive techniques are required to clarify many of these points. However, irrespective of their outcome, it appears abundantly clear that these complexes represent another unusual protein alteration found in the sera of certain patients with rheumatoid arthritis.

SUMMARY

The sera of 11 patients, 7 with advanced rheumatoid arthritis, 1 with questionable rheumatoid arthritis, and 3 with obscure disorders, showed large amounts of unusual γ-globulin complexes with sedimentation rates ranging from 9 to 17S. These were observed by direct ultracentrifugal analyses of whole serum as well as γ-globulin and euglobulin fractions. In some sera they made up most of the γ-globulin fractions.

The complexes were readily dissociated to 7S units in acid buffers and in 4 to 6 M urea. Addi-

tion of normal 7S γ-globulin to the sera and euglobulin fractions produced marked effects on the complexes. A pronounced shift to lower s-rate components was observed with disappearance of the larger 12 to 17S moieties.

The complexes were devoid of rheumatoid factor activity but were present in sera showing high titers in the serological reactions for rheumatoid factor. This relationship, however, was not a direct one. The complexes demonstrated strong inhibiting activity in certain of these reactions and were found to complex with rheumatoid factors, giving rise to components with s-rates as high as 28 to 31S. Some evidence was obtained for a relationship to "spontaneous precipitates."

The difficulties involved in distinguishing certain of these sera from those of Waldenström's macroglobulinemia and the importance of acid dissociation experiments are discussed.

The possibility is raised that these complexes might represent antigen-antibody complexes of the γ-globulin-anti-γ-globulin type. Evidence is presented suggesting that they are the 7S counterparts of the 19S rheumatoid factors combined with ordinary γ-globulin. Further observations with a more sensitive method of detection are required to determine the exact incidence and distribution of these complexes in rheumatoid arthritis and other diseases.

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EFFECTS OF PRIMAQUINE AND OTHER RELATED COMPOUNDS ON THE RED BLOOD CELL MEMBRANE. I. NA^+ AND K^+ PERMEABILITY IN NORMAL HUMAN CELLS *

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The important investigations of Carson, Dern and Beutler and their co-workers (1–8) have identified an inherent deficiency of erythrocyte glucose-6-phosphate dehydrogenase as the biochemical basis for the hemolytic anemia induced in "sensitive" individuals by administration of primaquine and certain related compounds. The relationship between this enzyme deficiency and the mechanism of the hemolytic response remains to be defined. The hemolysis certainly involves disruptive changes in the red cell membrane. The possibility of a direct action of primaquine on the membrane must be seriously considered. As Rothstein (9) has pointed out, it is important to examine the effects of toxic agents on the membranes of the cells as well as their effects on intracellular metabolism, since any administered agent will, of course, first contact the cell membrane.

In normal red blood cells, the studies of Beutler, Dern and Alving (5) indicate that hemolysis is produced *in vitro* only by concentrations of primaquine greatly in excess of pharmacologic blood levels. A prehemolytic loss of K^+ is, however, induced by subhemolytic concentrations of these agents. It was the purpose of the present investigations to study these *in vitro* prelytic abnormalities in red cell permeability.

It is accepted that the event of hemolysis occurring within a population of red cells represents an "all or none" response of individual cells, although the degree of hemolglobin loss by the individual cell varies with the lytic stimulus (10). In the case of prelytic phenomena involving changes in erythrocyte permeability, manifested by loss of K^+, red blood cells may respond in a heterogeneous fashion, as discussed by Ponder and Cox (11, 12). Such a heterogeneous response suggests the possibility of an all or none response on the part of some of the red cells as an explanation for a portion of the prelytic loss of K^+. Very significant contributions to the problem of individual red cell behavior were made by Passow and Tillman (13) in their studies of the prelytic loss of K^+ from human red blood cells exposed to Pb^{++} and by Eckel (14, 15) who has studied the exchangeable K^+ compartments of red cells treated with sodium fluoride. The latter investigator demonstrated a rapidly exchangeable K^+ compartment as well as a more slowly exchangeable K^+ compartment within the red cell sample and suggested that the rapidly exchangeable compartment represented complete K^+ permeability in some of the cells. Passow, Rothstein and Loewenstein (16), working with yeast cells, have also demonstrated that the loss of K^+ following exposure to a variety of basic redox dyes occurs as an all or none event for individual cells. In the present investigation, the prelytic loss of K^+ and gain of Na^+ induced by primaquine, methylene blue, and acetylphenylhydrazine was studied by analyzing dose-response and time-response relationships as well as osmotic fragility changes at varying times and drug concentrations.

METHODS

The blood samples from hematologically normal adult male and female donors were defibrinated with glass beads. The blood was centrifuged at $1{,}750 \times G$ for 10 minutes and the buffy coat removed by aspiration. The red cells were resuspended in their own serum. This suspension will hereafter be referred to as whole blood.

The whole blood was incubated in Warburg vessels

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with varying concentrations of primaquine diphosphate,[1] methylene blue, or acetylphenylhydrazine. Concentrations of each agent, 10 times the final desired concentration, were prepared in isotonic sodium chloride solution, adjusted to pH 7.4 or 7.8 with dilute NaOH and added to 9 vol of the whole blood. The hematocrits of the experimental preparations were adjusted to between 35 and 40 per cent. Concentrations of primaquine are expressed in moles per liter of whole blood, since the primaquine was rapidly distributed into both the red cell fraction and the serum.

Serum samples were removed at varying time intervals and the loss of K^+ was calculated as outlined by Ponder (17) and expressed as a percentage of the total K^+ content of the cells. K^+ and Na^+ determinations were made with a Beckman flame photometer. With the exception of a few experiments in which hemolysis ranged from 1 to 8 per cent, the great majority of the observations were made on samples in which no hemolysis had occurred.

Osmotic fragility determinations were performed according to the method of Parpart and colleagues (18). With severely damaged cells, some hemolysis occurred in 0.9 per cent NaCl, although there had been none in the whole blood. An additional tube containing 1.0 per cent NaCl was included in the fragility test to mimic the additional osmotic effect of the serum protein, and no additional hemolysis above that found in the serum itself was observed at this concentration of NaCl. Since estimation of per cent hemolysis on the basis of plasma hemoglobin measurements may introduce an error related to the amount of hemoglobin retained by individual hemolyzed or ghost cells, a more specific visual estimate of the percentage of hemolyzed cells was carried out in a manner somewhat similar to the original osmotic fragility studies of Whitby and Hynes (19), who used red blood cell counts. Stained cell preparations were made by placing 50 μl of the experimental cell suspensions into 10 ml of the 0.70 per cent NaCl-PO_4 buffer (pH 7.4) described by Parpart and co-workers (18) and allowing the injured cells to hemolyze during 20 minutes of incubation at room temperature. The lytic stimulus of 0.70 per cent NaCl was sufficient to lyse those cells which had a marked alteration in permeability, yet did not fragment the lysed cells beyond recognition, as did more dilute NaCl solutions. The cell suspension then was centrifuged, the supernatant aspirated and 1 ml of Dacie's solution (20) added. Coverslip smears then were prepared from the cell suspension in Dacie's solution. This solution was found to prevent destruction of the injured cells on contact with the glass slide. The smears were stained with Wright's stain and the percentage of ghosts in each preparation was estimated. Each estimate was based on microscopic counts of at least 500 cells from each of the 2 coverslips. The standard deviation of the percentage of ghost cells was $\pm$ 3.6 per cent, based on the pooled variance.

[1] The primaquine diphosphate was generously supplied by Dr. George D. Wessinger, Sterling-Winthrop Institute, Division of Sterling Drug, Inc., Rensselaer, N. Y.

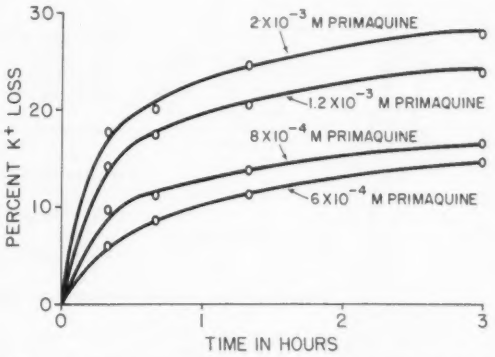

FIG. 1. TIME COURSE OF LOSS OF K^+ FROM NORMAL ERYTHROCYTES INDUCED BY VARYING CONCENTRATIONS OF PRIMAQUINE. Each point is the mean of duplicate determinations and represents the loss of K^+ expressed as a percentage of the total cellular K^+.

Primaquine-treated cells were stained for Heinz bodies, but the appearance and number of the latter correlated poorly with loss of K^+ and generally appeared at higher concentrations of primaquine than those concentrations producing loss of K^+. Consequently, no detailed study of Heinz bodies was conducted.

Lactic acid was measured by the method of Barker and Summerson (21) and reduced glutathione by the method of Grunert and Phillips (22) as modified by Beutler (7). Primaquine in concentrations below 1×10^{-3} M was measured by the method of Brodie, Udenfriend and Taggart (23) while above 1×10^{-3} M, the color of a metaphosphoric acid extract of serum or red blood cells was measured directly at 540 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. Percentage hemolysis was calculated by the method of Young, Izzo, Altman and Swisher (24).

RESULTS

Time course. The time course of the loss of K^+ from primaquine-treated red cells, illustrated in Figure 1, consisted of two distinct phases. The initial loss which occurred over 30 minutes was relatively rapid and proceeded at a rate related to the concentration of primaquine in the system. After the first 30 minutes, the loss of K^+ proceeded more slowly for the remainder of the experiment (2.5 hours) at the same rate for all concentrations of primaquine tested. Measurements of the serum primaquine concentration made over 3 hours of incubation, starting with a concentration of 3×10^{-3} M, revealed a rapid uptake of the drug by

TABLE I

*Determination of red cell Na^+ and K^+ **

	Time in Minutes	K+ Loss mM/l RBC	Na+ Gain mM/l RBC
6×10^{-4} M Primaquine	20	4.1	3.2
	40	5.9	5.2
	80	7.8	---
	180	10.1	9.0
8×10^{-4} M Primaquine	20	6.7	3.0
	40	7.8	8.0
	80	9.4	---
	180	10.6	9.0
1.2×10^{-3} M Primaquine	20	9.7	8.0
	40	12.0	9.5
	80	14.1	11.5
	180	16.5	19.5
2.0×10^{-3} M Primaquine	20	11.9	9.2
	40	13.3	12.5
	80	16.4	15.5
	180	18.8	----

* These values represent simultaneous measurements of the Na^+ and K^+ content of red cells incubated with varying concentrations of primaquine for the time intervals indicated. Each value is the mean of duplicate determinations.

cells during the first 20 minutes with only a slight further additional uptake during the rest of 2 hours' incubation. The time course of primaquine uptake was that of K^+ loss. After 30 minutes of incubation, the concentration of drug in the cells was twice that in the medium. The ratio of the cellular to plasma concentrations increased to 2.52 in 1 hour and 2.61 in 2 hours. The uptake of drug was slightly less at pH 7.4 than at pH 7.8. In all cases, the drug was concentrated by the cells to some extent, perhaps because of binding to cellular ligands.

Na^+. A comparison of the amounts of Na^+ gained by the red cells with the amounts of K^+ lost was based on simultaneous determinations of red cell Na^+ content and serum K^+. These results are summarized in Table I. Allowing for the relatively large error (5 to 15 per cent) in determinations of red cell Na^+ introduced by intracellular trapped plasma, it appears that there is a 1 : 1 exchange of Na^+ for K^+, as the latter leaks out of the cells.

Concentration of primaquine. Studies of the relationship between loss of K^+ and concentration of primaquine were carried out over the concentration range (2×10^{-4} M to 4×10^{-3} M) in which primaquine induced a prelytic loss of K^+ by the red cells. A few studies were performed, employing higher concentrations, that induced hemolysis. In Figure 2, the loss of K^+ and amount of hemolysis are plotted against the $\log_{10}$ of the primaquine concentration. Hemolysis occurred at concentrations of drug in excess of 5×10^{-3} M when the red cells were suspended in serum; when the red cells were suspended in 0.154 M NaCl solution, hemolysis appeared above 1×10^{-3} M. These latter findings conform with those presented by Beutler and colleagues (5). With primaquine concentrations above 2×10^{-2} M in the test system, useful information could not be obtained be-

cause of the formation of a gel of the cell suspension, preventing separation of the serum from the cells. With drug concentrations producing less than 70 per cent hemolysis, however, the amount of hemolysis was related to the $\log_{10}$ of the primaquine concentration in a normal distribution function. The loss of K^+ appeared at much lower concentrations of drug than those producing hemolysis, although the two curves of Figure 2 overlap to some extent. At a drug concentration of 5×10^{-3} M, no hemolysis was produced, but more than 50 per cent of the K^+ was lost. The slope of the curve relating loss of K^+ and drug concentration was relatively shallow compared with the curve relating hemolysis and drug concentration. Nevertheless, over the range of primaquine concentrations that could be investigated, the slope of the curve describing loss of K^+ resembles the lower half of a log-probability relationship. This relationship is similar to that seen in curves describing a log dose mortality relationship (25). The prelytic volume of the cells treated by primaquine ranged from 1.1 to 1.2 times the control volume. These prelytic red cell volumes were measured by hematocrit determinations as described by Ponder (26).

Osmotic fragility. Osmotic fragility studies were carried out in order to determine whether changes in fragility could be correlated with alterations in red cell permeability, as manifested by loss of K^+. Figure 3 is a diagram of osmotic fragility tests obtained after exposing aliquots of a whole blood sample to 8×10^{-4} M primaquine for 180 minutes or to 2×10^{-3} M primaquine for 40, 80 and 180 minutes. Figure 3 also includes an osmotic fragility curve obtained after incubation for 180 minutes of a control aliquot from the same blood sample in the absence of primaquine. In neither of these concentrations of drug was any hemolysis produced in the whole blood. The osmotic fragility patterns produced by these two concentrations of drug clearly illustrate two types of induced changes in the erythrocytes. At the lower drug concentration, 8×10^{-4} M, increased fragility of the whole cell population is evident and the entire curve is shifted symmetrically to the left. At the higher drug concentration, however, in addition to the symmetrical shift of the upper portion of the curve there is an evident upward shift of the curve from the baseline, increasing with time. This change in osmotic fragility is indicative of a profound change in the membrane of some of the cells, which makes them readily susceptible to osmotic lysis.

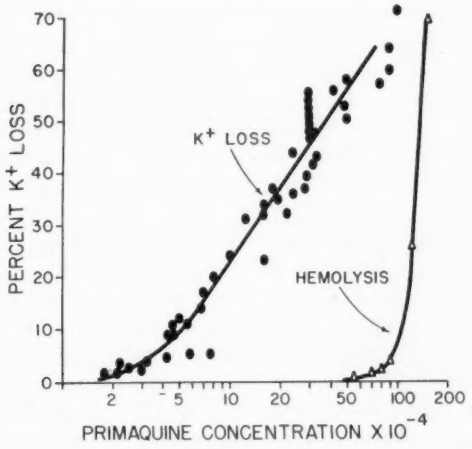

FIG. 2. RELATIONSHIP BETWEEN PERCENTAGE LOSS OF K^+ AND CONCENTRATION OF PRIMAQUINE. The abscissa represents the $\log_{10}$ of the primaquine concentration in moles per liter of whole blood and the ordinate indicates the percentage loss of K^+ by red cells after 2 hours of incubation with primaquine.

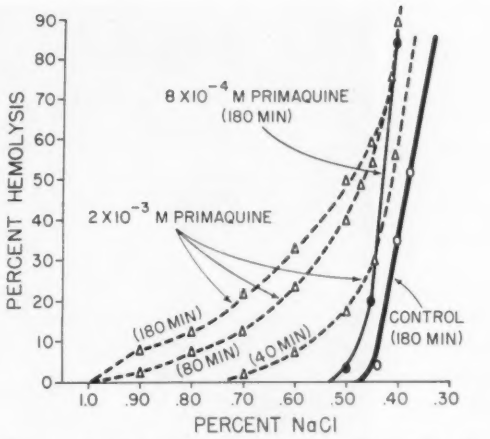

FIG. 3. OSMOTIC FRAGILITY STUDIES. The graph illustrates a representative set of results obtained, utilizing aliquots of the same red cell sample. The osmotic fragility studies were conducted on a control sample incubated without primaquine, on a sample incubated with 8×10^{-4} M primaquine and on three samples incubated with 2×10^{-3} M primaquine, for the time intervals indicated.

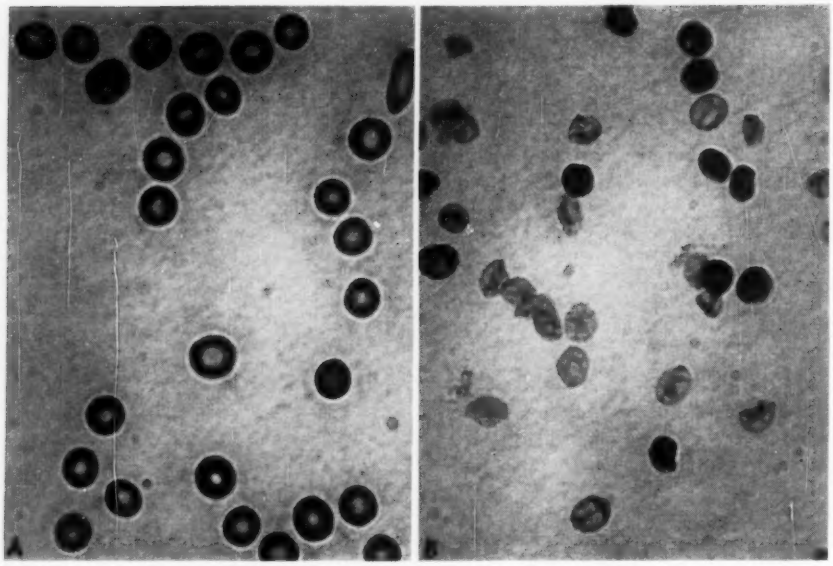

FIG. 4. ERYTHROCYTE GHOSTS PREPARED BY OSMOTIC LYSIS OF CELLS PREVIOUSLY TREATED WITH PRIMAQUINE. The red cells were hemolyzed in 0.70 per cent NaCl after incubation with the drug; 4A is a photograph of a control sample incubated without primaquine and then exposed to 0.70 per cent NaCl; 4B demonstrates a sample exposed to 3×10^{-3} M primaquine with 50 per cent loss of K^+ and production of 50 per cent ghosts after exposure to 0.70 per cent NaCl.

Osmotic fragility studies employing staining of erythrocyte ghosts. Although the osmotic fragility studies suggested that some of the K^+ was lost from the primaquine-treated red cells in an "all or none" manner, more direct confirmation of this point was sought by incubating red cells with primaquine, placing them in 0.70 per cent NaCl to produce lysis of those cells with damaged membranes, and staining the entire sample to visualize the hemolyzed ghost cells. Figure 4 illustrates two of these stained red cell ghost preparations; Figure 4A was prepared from a control sample of blood without primaquine which was incubated and treated in the same fashion as was a primaquine-treated sample with 50 per cent ghosts, shown in 4B.

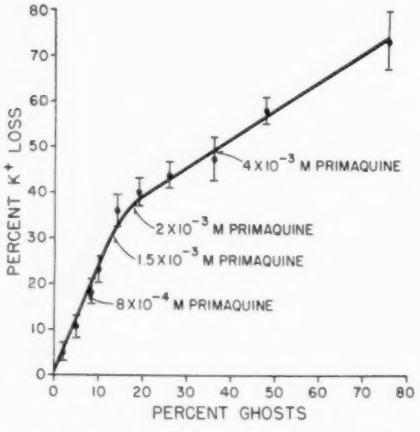

FIG. 5. RELATIONSHIP OF PERCENTAGE LOSS OF K^+ INDUCED BY PRIMAQUINE TO PERCENTAGE GHOSTS PRODUCED BY SUBSEQUENT EXPOSURE TO HYPOTONIC SODIUM CHLORIDE. The standard deviation about the mean value for each point is indicated on the graph.

Figure 5 represents a graph of the percentage of ghosts cells versus the percentage loss of K^+. Although increased cation permeability is associated with increased osmotic fragility, if loss of K^+ occurred only as an all or none phenomenon in individual cells, one might expect a linear 1 : 1 relationship between loss of K^+ and the proportion of ghost cells. A simple relationship of this type was not found. Rather, the curve of Figure 5 appears to be a composite of two lines of different slopes. At lower concentrations of primaquine, the percentage loss of K^+ induced was greater than the percentage of cells hemolyzed by hypotonic

saline. Thus the K^+ released was greater than that which could have come from only those cells with markedly abnormal membranes.[2] The upper part of the curve, however, shows a loss of K^+ more nearly directly proportional to the percentage of ghosts in the sample. These data suggest a mixed response of a population of primaquine-treated erythrocytes—some loss of K^+ occurs from all of the cells, and a complete release of K^+ occurs in other cells, with the all or none response predominant at higher concentrations of primaquine. Such an analysis of the relationship of the loss of K^+ and lysis of cells is consistent with the two types of osmotic fragility response shown in Figure 3. This interpretation is further supported by the fact that the inflection point of the curve of Figure 5 at 30 per cent loss of K^+ occurs at a concentration of 1.5×10^{-3} M primaquine, a concentration of drug between those which produce the two types of osmotic fragility response.

Metabolic observations. Metabolic disturbances which might account for K^+ loss could not be found in primaquine-treated red cells. Lactic acid production during 2 hours of incubation of normal erythrocytes and red cells incubated with varying concentrations of primaquine was compared. Normal red cells produced 17.0 ± 6.0 (SD) mg lactic acid per 100 ml RBC per 2 hours, while red cells incubated with 1×10^{-3} M primaquine produced 14.9 ± 1.1 (SD) mg per 100 ml and cells exposed to 3×10^{-3} M primaquine produced 17.7 ± 5.3 (SD) mg per 100 ml during the same period. There are no significant differences among these results.

The reduced glutathione content of red blood cells from six normal individuals remained at normal levels after the cells were incubated with concentrations of primaquine that caused large losses of K^+. The mean pre-incubation value for reduced glutathione was 62.9 ± 19.7 mg per 100 ml of normal red blood cells. Aliquots of the same red cell samples after 2 hours' incubation at 37° C in the absence of primaquine were found to contain 70.6 ± 16 mg per 100 ml of reduced glutathione. After 2 hours' incubation with 1×10^{-3} M primaquine, a value of 72.7 ± 13.0 mg per 100 ml of reduced glutathione was obtained, and after incubation with 3×10^{-3} M primaquine, 61.7 ± 10.7 mg per 100 ml of reduced glutathione was present.

Methemoglobin formation was measured in red cell samples after 2 hours of incubation and found to equal 2 per cent of the total hemoglobin present in untreated samples. The same value was found for red cell samples treated with 2, 5 or 10×10^{-4} M concentrations of primaquine, concentrations which result in significant loss of K^+. Treatment with a concentration of 2×10^{-3} M primaquine, which induces a 50 per cent loss of K^+, resulted in an increase in amounts of methemoglobin to 8 to 12 per cent of the total hemoglobin after 2 hours of incubation.

pH. The loss of K^+ induced by treatment of erythrocytes with 3×10^{-3} M primaquine was greater at pH 7.8 than at pH 7.4; 51.3 per cent ± 6.7 ($N = 15$) of the cellular K^+ is lost at the higher pH compared with a loss of 38.4 per cent ± 7.7 ($N = 6$) of the K^+ at pH 7.4.

Effect of serum. Primaquine treatment of the erythrocytes in normal serum at pH 7.8 was found to augment significantly the loss of K^+ compared with red cells treated in 0.154 M NaCl solution. Primaquine at concentrations of 3×10^{-3} M in serum resulted in the loss of 51.3 per cent of the cellular K^+; the same concentration of drug in

[2] Although the initial portion of Figure 5, indicating percentage loss of K^+ in excess of the number of destroyed cells, conceivably could be explained by the breakdown of a small number of cells having much more than normal content of K^+, rather than some loss of K^+ by all cells, this is extremely unlikely on a theoretical basis. Utilizing the recent values for mean corpuscular volume obtained through study of individual erythrocytes by Houchin, Munn and Parnell (27), it can be shown that 99.99 per cent of normal erythrocytes examined by them had a volume less than 1.24 times the mean volume (3 SD from the mean). Turning to the data presented in Figure 5, however, it is apparent that if there were no loss of K^+ by all cells, 10 per cent of the cells would have to lose 22 per cent of the total K^+ content of the red cell sample. This would require that the K^+ content of the 10 per cent of hemolyzed cells was approximately 300 mmoles per L of RBC water. Calculation of the obligate increase in cell water for this theoretical situation, taking into account Ponder's correction for the deviation in behavior of the normal red cell from that of a perfect osmometer (28), would indicate a 1.8-fold increase in cell water or 1.26-fold increase in cell volume. As shown above, however, less than 0.01 per cent of the cells has a volume 1.26 times the mean volume, since 99.99 per cent have a volume less than 1.24 times the mean. This demonstrates that some of the loss of K^+ indicated by the first portion of the curve in Figure 5 represented a loss greater than that accounted for by individual cell breakdown.

saline resulted in the mean loss of only 35.7 per cent. The mean difference in loss of K^+ between 12 paired samples was 14.2 per cent ± 8.4 (2 SD). This difference is highly significant. A similar difference, although less marked, was also observed in tests carried out at pH 7.4. Heat inactivation of the serum at 56° C for 30 minutes did not alter this effect nor did the addition of ethylenediamine tetraacetate in concentrations from 1×10^{-4} M to 9×10^{-3} M to the serum. The addition of physiological concentrations of Ca^{++}, Mg^{++}, or Zn^{++}, alone and in combination, to the samples treated in NaCl solution did not result in increased loss of K^+.

Other agents. The effects of other agents, including methylene blue and acetylphenylhydrazine, on the loss of erythrocyte K^+ was evaluated. These agents were studied in systems at a pH of 7.8. Methylene blue induced 0 to 100 per cent loss of K^+ over a concentration range extending from 4×10^{-4} M to 6×10^{-3} M, when the red cells were suspended in serum. Acetylphenylhydrazine in serum produced a similar effect at concentrations between 3×10^{-4} and 2.4×10^{-2} M. Both of these agents, when studied *in vitro* like primaquine, demonstrated a dual relationship between loss of K^+ and osmotic fragility, and both also exerted their effects on loss of K^+ more readily in serum than in NaCl solution.

DISCUSSION

Based on the time-response and osmotic fragility data presented, the *in vitro* prelytic loss of K^+ induced by primaquine, methylene blue and acetylphenylhydrazine is composed of two fractions. A smaller loss of K^+, occurring at lower concentrations of primaquine, appears to affect all cells uniformly. In addition, depending on the drug concentration, there may be an all or none loss of K^+ involving just a portion of the red cell population. The all or none type of response found is consistent with the observations of Passow and co-workers (16) who found that methylene blue induced a loss of K^+ from yeast cells as an "all or none" phenomenon. In addition, the finding of two types of loss of K^+ by red cells agrees with similar findings by Passow and Tillman (13), who studied the effects of Pb^{++} on red cells, and by Eckel (14, 15) who studied fluoride-poisoned red cells.

The osmotic fragility tests on the red blood cells incubated with primaquine indicated a decreased resistance to osmotic lysis in contradistinction to the work of Passow and Tillman (13) which showed an increased resistance to osmotic lysis of human red cells treated with Pb^{++}, although such cells had also lost K^+ in a fashion analogous to the cells treated with primaquine. One explanation for this apparent discrepancy may be found in the work of Vincent and Blackburn (29, 30) who also studied Pb^{++}-induced loss of K^+ from red blood cells. Vincent found that an initial loss of K^+ from the cells was not accompanied by a simultaneous inward movement of Na^+; rather, movement of Na^+ followed some time later. This observation suggests that the observed decreased resistance to osmotic lysis of red cells treated with primaquine was related to a general increase in cation permeability, as illustrated by the entry of Na^+ concomitant with loss of K^+. By contrast, entry of Na^+ appears to be delayed in cells treated with Pb^{++}, making them shrink and become osmotically resistant. Although differing in direction of shift, the pattern of alteration in osmotic fragility induced by primaquine is compatible with the patterns found by Passow and Tillman (13) and Eckel (14, 15), which these authors interpreted as evidence of an "all or none" response by segments of a red cell population. In addition, the finding in the present investigation of a symmetrical shift of the entire osmotic fragility curve with time also correlates well with the observations of these other investigators; the symmetrical shift in osmotic fragility can be interpreted similarly to represent some increased permeability of all of the remaining cells.

Although lactate production and reduced glutathione content can be considered only gross measures of glycolytic integrity, the very rapid appearance of the all or none loss of K^+, coupled with the lack of effect of primaquine on lactate production or reduced glutathione content, suggests that *in vitro* effects of primaquine may be physicochemical effects on the membrane rather than interference with metabolically determined K^+ gradients. Additional evidence for such a direct effect on the red cell membrane lies in the fact that the critical hemolytic volume of red cells exposed to primaquine is only 110 to 120 per cent of their normal volume, indicating a distinct decrease in

the ability of treated red cells to resist hemolysis in the face of swelling, as pointed out by Ponder (26).

Reduced glutathione content of primaquine-treated red cells was investigated because Benesch and Benesch (31), Sheets, Hamilton and DeGowin (32) and Fegler (33) all have called attention to the importance of sulfhydryl (SH) groups for maintenance of red cell integrity, and Eichel (34) and Woodward (35) have found reduced glutathione to constitute approximately 95 per cent of the nonprotein sulfhydryl content of red cells. Passow and colleagues (16) have suggested that the loss of K^+ from yeast, induced by basic aniline dyes, may be related to alteration of SH groups by these oxidizing agents. Beutler and colleagues (6, 7), however, have pointed out that normal red blood cells are able to maintain their reduced glutathione in the face of 500 mg per 100 ml acetylphenylhydrazine, a concentration found to produce leakage of 100 per cent of cellular K^+ in our studies. Therefore, it appears that the loss of K^+ induced in normal red cells by acetylphenylhydrazine and primaquine is not related to any alteration in the total reduced glutathione content of the cells, since the loss of K^+ is produced by concentrations of primaquine that do not measurably affect the total reduced glutathione content of the cells. A critical, but numerically small, alteration by primaquine of SH groups in the membrane itself, however, cannot be excluded.

The reason for the marked enhancement of the primaquine-induced loss of red cell K^+ by an increase in pH from 7.4 to 7.8 is not clear but may be related in part to the moderate increase in uptake of primaquine by the red cells at the higher pH. In addition, as Emerson, Ham and Castle (36, 37) have pointed out, the oxidizing properties of these resonating compounds is enhanced by increased pH, and they would be expected to exert a more marked effect under these circumstances.

One of the most interesting, although unexplained, observations is the enhancing effect of serum on the loss of K^+ induced by all three agents studied. The enhancing effect of serum on aniline-induced hemolysis has been known since it was first described by Browning and Mackie in 1914 (38) and again in 1933 by Ponder (39, 40). Ponder (41) has pointed out that the aniline dye-red cell system alone differed from other hemolytic systems he had studied in that the action of all other hemolytic agents, except the aniline compounds, is inhibited by the presence of serum. While serum enhanced the loss of K^+ induced by primaquine, methylene blue and acetylphenylhydrazine in the present study, the enhancing effect was unaltered by the addition of chelating agents, heat treatment or the addition of cations to the saline system. These observations seem to exclude activities of complement or divalent cations in this effect. The amount of primaquine taken up by the cells was essentially the same or slightly greater in a saline-containing system, so that the effect of serum is apparently not related to differences in distribution of primaquine.

SUMMARY

1. Primaquine diphosphate induces an *in vitro* prehemolytic loss of K^+ from normal human red blood cells over a concentration range from 2×10^{-4} M to 5×10^{-3} M primaquine per L of whole blood. Above 5×10^{-3} M, hemolysis begins to appear.

2. The time course of the prelytic loss of K^+, the alterations in osmotic fragility and evidence provided by a technique for visual observation of the affected cells, all indicate that in addition to some loss of K^+ by all the cells in a sample in the presence of higher concentrations of primaquine, there is a portion of the red cell population which develops an "all or none" complete permeability to K^+. The proportion of loss of K^+ due to the latter mechanism is dependent upon the primaquine concentration.

3. Concentrations of primaquine that induce loss of K^+ by red cells do not affect lactic acid production over a 2 hour period of incubation. Similarly, the reduced glutathione content of normal red cells is unaffected by their exposure for 2 hours to concentrations of primaquine that cause significant loss of K^+. Significant loss of K^+ can also be induced by concentrations of primaquine that produce no methemoglobinemia; at drug concentrations producing 50 per cent loss of K^+, 8 to 12 per cent methemoglobin can be detected.

4. The presence of serum in the incubation system distinctly enhances the loss of K^+ induced by a given concentration of primaquine. In addition,

the magnitude of the loss of K^+ at a given primaquine concentration varies directly with the pH of the whole blood.

5. Methylene blue and acetylphenylhydrazine induce a similar prelytic loss of K^+, and like primaquine, these agents exert a greater effect in the presence of serum.

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EFFECTS OF PRIMAQUINE ON THE RED BLOOD CELL MEMBRANE. II. K^+ PERMEABILITY IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT ERYTHROCYTES *

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An unexplained anomaly of "primaquine-sensitive" erythrocytes is their ability to resist hemolysis *in vitro*, even when exposed to concentrations of primaquine that are greatly in excess of pharmacologic levels. Beutler, Dern and Alving (1) have studied *in vitro* hemolysis of primaquine-sensitive red cells in detail and demonstrated that it occurs between 1.5 and 4×10^{-3} M primaquine (0.6 to 2.0 mg per ml) when measured in a test system containing saline buffered at pH 7.0. They were unable to demonstrate any difference between normal and abnormal cells with respect to the drug concentration required to produce *in vitro* hemolysis. *In vitro* studies on normal cells, reported in an accompanying paper (2) generally demonstrated no hemolysis with concentrations of less than 5×10^{-3} M primaquine; these observations were made on whole defibrinated blood, and when compared with Beutler's studies of red cells suspended in buffered saline, it appears that serum proteins inhibited hemolysis of red cells exposed to concentrations of primaquine less than 5×10^{-3} M.

In comparing *in vitro* observations on hemolysis produced by primaquine, with the *in vivo* effects of the drug, Beutler and colleagues have (1) suggested that the drug, or one of its metabolic by-products, damages the sensitive erythrocytes, thereby making them susceptible to removal by *in vitro* mechanisms but not to *in vitro* hemolysis. Brodie and Udenfriend (3), who studied the hemolytic effect of primaquine, suggested that a metabolic product of that drug was an agent capable of damaging red cells which might even produce *in vitro* hemolysis.

Previous studies (2) have demonstrated that prelytic increased cation permeability induced in normal erythrocytes by primaquine is a more sensitive indicator of *in vitro* damage to the cells than is hemolysis. This increased permeability, manifested by loss of K^+ from normal erythrocytes is produced by concentrations of primaquine or acetylphenylhydrazine which are 3×10^{-4} M or greater. These *in vitro* concentrations of primaquine appear to have serious deleterious effect on the red cell membrane. Desforges, Kalaw and Gilchrist (4) have demonstrated noncompetitive inhibition of glucose-6-phosphate dehydrogenase by 1×10^{-3} M primaquine. Mohler and Williams (5) have demonstrated ATP instability in blood incubated with 3.4×10^{-3} M phenylhydrazine, and Jandl and Allen (6) have demonstrated oxidative denaturation of hemoglobin in intact red cells following production of methemoglobin *in vitro* by phenylhydrazine. The concentrations of these agents necessary to produce *in vitro* glucose-6-phosphate dehydrogenase inhibition, ATP breakdown or methemoglobin are, however, far in excess of those which will induce loss of K^+ from intact cells (2).

Loss of K^+ in itself is, of course, merely an indication of cell membrane damage but its occurrence serves to emphasize that a variety of deleterious effects is produced *in vitro* by concentrations of drug of 1×10^{-3} M or greater. Since loss of K^+ occurs at lower concentrations of drug than those needed to produce other demonstrated *in vitro* changes, it appeared to be worthwhile to carry out similar studies of the *in vitro* cation permeability of red blood cells from patients with primaquine-sensitive cells. These studies were

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undertaken to investigate the possibility that the loss of K^+ might be a more sensitive indication of an *in vitro* difference between these cells and normal cells.

METHODS

Blood samples were obtained from 7 individuals who were found to have deficient activity of glucose-6-phosphate dehydrogenase in their red cells. Five of these were healthy Negro males with normal hematocrits and no evident disease at the time of the experiments. Blood from one Caucasian female of Sicilian extraction whose glutathione stability test placed her in the "intermediate" group (5) was also tested. In addition, blood was obtained from a sixth Negro male during the course of a Furadantin-induced hemolytic episode. During this episode, the patient's hematocrit dropped from 40 to 29 per cent; at the time when the *in vitro* experiments were conducted, his hematocrit was 35 per cent and he was still receiving the drug, indicating that active *in vivo* destruction of this patient's red blood cells was occurring at the time of the study.

All the blood samples were subjected to the screening test of Motulsky, Kraut, Thieme and Musto (7) for decreased glucose-6-phosphate dehydrogenase activity and found to be abnormal. Glutathione stability tests were conducted as outlined by Beutler (8) and demonstrated abnormally low, postincubation reduced glutathione content of the erythrocytes of all the donors except for the female patient. This patient's reduced glutathione values were 48.5 mg per 100 ml initially and 27.5 mg per 100 ml after incubation with acetylphenylhydrazine.

The conditions of *in vitro* incubation of erythrocytes with primaquine and techniques for measuring loss of K^+, osmotic fragility, reduced glutathione, primaquine and hemolysis are described in an accompanying paper (2). The *in vitro* concentrations of primaquine[1] employed ranged from 1×10^{-4} to 3×10^{-3} M. Loss of K^+ after *in vitro* exposure of red cells to the drug was studied on samples of blood from 3 primaquine-sensitive individuals and 1 individual whose glutathione stability test placed her in the intermediate group.

In an effort to evaluate the role of metabolic products of primaquine, experiments were conducted in which red cells from 2 primaquine-sensitive individuals were incubated with serologically compatible serum from 2 normal individuals who had ingested primaquine diphosphate, 30 mg per day, for 3 to 5 days, the last dose being taken 4 hours prior to donation of the serum. The concentration of primaquine present in these sera ranged from 1 to 3×10^{-4} M. The primaquine-containing sera were incubated with autogenous red cells, and with serologically compatible cells from 2 of the glucose-6-phosphate deficient individuals. Sera obtained from the normal donors prior to ingestion of primaquine were also incubated with the abnormal cells. Incubations without added glucose in the system were extended to 20 hours in the hope of accentuating any differences between normal and abnormal erythrocytes.

[1] The primaquine diphosphate was generously supplied by Dr. George D. Wessinger, Sterling-Winthrop Institute, Division of Sterling Drug, Inc., Rensselaer, N. Y.

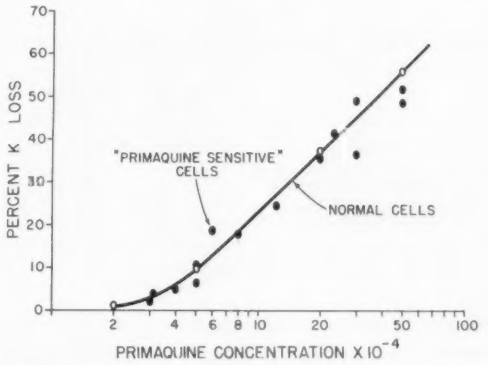

FIG. 1. PERCENTAGE LOSS OF K^+ BY "PRIMAQUINE-SENSITIVE" ERYTHROCYTES INCUBATED WITH VARYING CONCENTRATIONS OF PRIMAQUINE. The mean percentage loss of K^+ by normal red cells in response to varying concentrations of primaquine is indicated by the line connecting the open circles which are representative points taken from the accompanying paper (2). The solid dots represent the experimental results obtained by utilizing primaquine-sensitive red cells.

RESULTS

Incubation of "sensitive" erythrocytes with primaquine. The abnormal red cells were incubated with primaquine in a range of concentrations described above, and in Figure 1 the results of 2-hour incubation studies are compared with similar results obtained from study of normal cells. It is evident, under these circumstances, that the abnormal cells behave no differently from the normal cells and apparently require the same *in vitro* dosage of primaquine to induce prelytic loss of K^+.

Short-term incubation (up to 5 hours) of normal or primaquine-sensitive red cells, either in their own serum or in the serum from normal donors who had taken the drug, produced no significant loss of K^+ or hemolysis. Incubation of these combinations of red cells and serum for 20 hours, in the absence of glucose, yielded the results shown in Table I.

In addition, incubation of a whole blood sample taken from the individual with active hemolytic

TABLE I

*Per cent loss of K^+ induced by 20 hours of in vitro incubation**

	Abnormal red cells	Normal red cells
Autologous serum	28.2, 27	36, 33, 24
Compatible donor serum before ingestion of primaquine	31	38.6, 36
Compatible donor serum after ingestion of primaquine	33.7, 26.2	32.6, 35.6

* Each value is the mean of duplicate determinations.

disease caused by nitrofurantoin (Furadantin), presumably containing Furadantin or its potentially hemolytic metabolites, yielded an 18 hour loss of K^+ of 23.3 per cent with 1 per cent hemolysis.

It is obvious that the abnormal red cells in the presence of primaquine- or Furadantin-containing serum lost no more potassium than did normal red cells, incubated for a like period of time. The normal result obtained on the blood of the patient already undergoing drug-induced *in vivo* hemolysis suggested that destruction of the patient's cells ceased when the blood was removed from the body. All of the values for loss of K^+ from both normal and abnormal red blood cells are consistent with Ponder's observation on loss of K^+ by red cells incubated at 37° C in the absence of glucose (9). Osmotic fragility studies of the abnormal red cells, both initially and after incubation with either added primaquine or primaquine-containing serum, were comparable with those of normal cells treated in similar fashion.

DISCUSSION

The results of these studies on red cell K^+ permeability are entirely consistent with the observations of Beutler, Dern and Alving (1), which point out the similarity of normal and primaquine-sensitive red cells with regard to primaquine-induced *in vitro* hemolysis. They are also consistent with experiments performed by Feldman, Packer, Murphy and Watson (10) who incubated red cells from a sensitive individual with pamaquine-containing plasma from a normal individual and failed to produce hemolysis of the sensitive cells. In spite of the fact that loss of K^+ is a considerably more sensitive parameter of red cell damage than is hemolysis, there is no demonstrable evidence of a difference in this respect between normal and glucose-6-phosphate dehydrogenase deficient erythrocytes exposed to primaquine or to presumed primaquine metabolites in an *in vitro* test system.

These observations emphasize the difficulty in drawing conclusions about primaquine-sensitive erythrocytes from *in vitro* studies and strongly re-emphasize the point made by Beutler, Dern and Alving (1) that *in vivo* mechanisms of red cell destruction are an essential part of the clinical picture of primaquine-induced hemolytic anemia. The need for more investigation in this area is clearly indicated, with emphasis on the relationship between *in vivo* removal mechanisms and the metabolic defect in the cells.

SUMMARY AND CONCLUSIONS

1. Erythrocytes which are deficient in their glucose-6-phosphate dehydrogenase activity manifest prelytic loss of K^+ identical with that of normal cells when exposed to primaquine *in vitro*.

2. Serum from normal individuals taking 30 mg of primaquine daily, when incubated with primaquine-sensitive erythrocytes from the patients, failed to induce a loss of K^+ which differed from that of normal red cells incubated over the same 20 hour period.

3. Altered K^+ permeability in both normal and primaquine-sensitive erythrocytes occurs at concentrations of drug below those which produce various biochemical abnormalities, suggesting that primaquine and other related compounds may sequentially affect the erythrocyte membrane in a critical fashion before altering intracellular biochemical processes.

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THE INFLUENCE OF VASOPRESSIN ON THE PERMEABILITY OF THE MAMMALIAN COLLECTING DUCT TO UREA*

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The efficient operation of the renal concentrating mechanism is dependent upon the action of vasopressin on the permeability of the distal convoluted tubule to water (1). In the presence of this hormone, hypotonic fluid entering the distal convolution equilibrates osmotically with fluid in the surrounding renal cortical tissue, and isotonicity of the fluid delivered to the collecting ducts is achieved (1, 2). The collecting ducts are surrounded by a medullary interstitium which is variably hyperosmolal to plasma (3–5). The osmotic movement of water from collecting duct lumen to medullary interstitium results in the excretion of a hypertonic urine. Despite evidence that movement of water across the collecting duct epithelium may occur in the apparent absence of vasopressin (6, 7), most schemes of the urinary concentrating process include the hypothesis that water permeability in this segment of the nephron is enhanced by this hormone. The present study was designed to test that hypothesis.

Kinetic analysis of the action of vasopressin on the movement of water and solute across the toad skin indicates that the hormone acts to increase the pore size in this epithelial membrane (8, 9). If vasopressin exerts a similar action on the collecting duct epithelium, the permeability of the membrane to various partially permeant solutes should be affected by this agent. Since the urea concentration within the collecting duct fluid rises as water is abstracted from the lumen (10), it may be concluded that this membrane offers a barrier to the free diffusion of this molecule. It was therefore anticipated that an effect of vasopressin on the permeability of the collecting duct epithelial membrane would be manifested by an increased rate of movement of urea across this membrane. The experiments to be described indicate that vasopressin does augment the movement of urea from the collecting duct lumen to the medullary interstitial fluid. The present results are in accord with the recent report of Maffly, Hays, Lamdin and Leaf (11) which demonstrates that vasopressin markedly increases the permeability of the isolated toad bladder to urea.

The studies of Schmidt-Nielsen and O'Dell have suggested the presence of a specific transport mechanism for urea within the distal tubular system of the sheep (12). As discussed by Levinsky and Berliner (13), however, the present experimental evidence in the dog is entirely compatible with the hypothesis that urea enters the medullary tissue space of this species by passive diffusion along a concentration gradient. Whereas the data to be presented in no way refute the possibility of active transport of urea in the dog nephron (or an effect of vasopressin on this hypothetical transport system), they are fully compatible with the concept of solely passive movement of urea.

METHODS AND PROCEDURES

All studies were performed in anesthetized mongrel dogs, in two distinct groups of experiments.

I. The relative concentrations of urea in the urine and in the medullary tissue were determined during the steady state. Studies were done at high urine flow rates, and at relatively low urine to plasma urea concentration ratios, so that the urea concentration in the urine would closely approximate that in the terminal portion of the collecting duct system.

The protocol of a representative experiment is outlined in Table I. Anesthesia was induced and maintained with thiopental sodium. Both ureters were catheterized through flank incisions, and urine was collected separately from each kidney. Following induction of anesthesia, an intravenous water load consisting of 2.5 per cent glucose in water, equivalent to 4 to 5 per

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TABLE I

Comparison of medullary and urinary urea concentrations during water diuresis and during the administration of vasopressin and mannitol; protocol of a single experiment *

	Right kidney				Left kidney			
	V	U_{urea}	U_{osm}	C_{in}	V	U_{urea}	U_{osm}	C_{in}
min	*ml/min*	*mmoles/L*	*mOsm/kg*	*ml/min*	*ml/min*	*mmoles/L*	*mOsm/kg*	*ml/min*
0	Anesthetized with 2.5% thiopental sodium							
5–30	Operation, both ureters catheterized							
10–55	Water load, 560 ml 2.5% glucose in water i.v.							
35	Infusion, inulin, 40 mg/ml at 0.25 ml/min							
50	Infusion, 140 mmoles/L urea in 2.5% glucose in water at 9.0 ml/min							
133–148	4.65	60.0	74	33.5	5.13	56.6	79	39.8
148–163	4.73	64.1	83	39.8	5.20	60.5	80	37.8
163–178	4.52	72.0	90	43.0	4.93	66.5	90	37.8
179	Left kidney removed							
185	Infusions changed to: 5% mannitol + 180 mmoles/L urea at 3.2 ml/min; 40 mg/ml inulin at 0.115 ml/min Pitressin, 400 mU i.v. immediately, and 50 mU/kg/hr							
315–330	6.77	49.1	227	37.4				
330–345	6.88	58.5	230	41.9				
345–360	6.46	60.5	238	45.0				
361	Right kidney removed							

* Dog Ka; weight, 14.1 kg.

cent of body weight, was given. Thereafter urea was infused in order to achieve relatively high urine and tissue concentrations, and to permit accurate determinations of urea in small tissue slices. When a water diuresis had been achieved, and the urine flow rate was essentially stable, three 15-minute clearance periods were collected from each kidney. Immediately after the termination of the third clearance period, the left renal pedicle was clamped and the kidney removed. Vasopressin (aqueous Pitressin; Parke, Davis) was then given, 300 to 400 mU intravenously immediately, and a sustaining infusion of 50 mU per kg per hour. Mannitol was infused in order to maintain a high urine flow rate from the remaining right kidney. Urea infusion was continued. At the time a relatively stable urine flow was achieved, 3 clearance periods were collected, at the termination of which the right kidney was removed. Inulin was given throughout the procedure by means of a constant infusion pump. In three experiments, as shown in Table III, the above procedure was reversed. Mannitol and vasopressin were given at the outset of the experiment, and after removal of one kidney, these were withdrawn, the animal was given a water load, and the second kidney was removed when a water diuresis was attained.

Immediately following removal, sections were taken from each kidney for analysis. Serial adjacent slices were cut from the papilla and inner medulla by methods previously described (4). These were homogenized in water and urea analyses were performed on the homogenates. Aliquots from each area were dried in an oven for the determination of the tissue water content. The urea concentration per unit of tissue water could then be calculated. Urea was determined in tissue homogenates and urine specimens by the Conway micro-diffusion method (14). Inulin was determined in urine and plasma by reaction with diphenylamine, following alkali digestion (15). The osmolality of urine and plasma was determined by the method of Bowman, Trantham and Caulfield (16). Statistical analysis of the data in Table III was performed by methods described by Snedecor (17).

II. In order to determine the effect of vasopressin on the flux rate of urea into the renal medulla, a second set of experiments has been performed. Paired studies were done during water diuresis and during the infusion of vasopressin and mannitol. As in the previous set of experiments, urea was added to each infusion and 2 to 3 clearance periods were collected when a stable urine flow was attained. Five minutes before the removal of each kidney, an intravenous infusion of C^{14}-labeled urea was started. Five urinary collection periods, each of 30 seconds' duration, preceded the removal of each kidney. The kidney was sectioned as described, except that in addition to sections from the papilla and inner medulla a slice of cortical tissue was also removed for analysis. Analyses for urea and water concentrations in the tissues were performed. In addition, an aliquot of each homogenate was counted in a liquid scintillation counter (Tri-Carb) for C^{14} activity. Urine specimens were similarly analyzed for urea and counted for C^{14}. Specific activities were calculated for tissue and urine specimens. All specimens from the second (vasopressin plus mannitol) part of the experiment were corrected for residual radioactivity by subtracting the specific activity of a urine collected immediately prior to the second infusion of isotope. This correction was minimized

by administering approximately twice as much isotope in the second infusion. Correction for quenching was achieved by the internal standard method. Following initial counting of urine and tissue specimens, a uniform amount of C^{14}-urea standard was added to each specimen, the counting procedure repeated, and the counts recorded compared with those from the same amount of standard added to an identical volume of water in the liquid phosphor system. Quenching was minimal (less than 5 per cent) in urine, medullary and papillary specimens, and appreciable only in cortical homogenates.

RESULTS

I. *The effect of vasopressin on the relative urea concentrations in the urine and in the renal medulla.* The ratio of the urea concentration in the inner medulla and papilla to that in the urine has been compared during water diuresis, and during the infusion of vasopressin and mannitol in 12 paired experiments. The protocol of a single representative experiment is shown in Table I, and the tissue analyses from this experiment are detailed in Table II. The highest tissue urea concentration (expressed as micromoles per gram of water) was always present in either the papillary slice or that from the immediately adjacent inner medullary zone (designated as "Inner medulla, 1" in Table II). By relating the highest tissue urea concentration to the urinary urea concentration in the clearance period immediately preceding nephrectomy, a tissue to urine concentration ratio for urea has been computed. In the experiment tabulated, this ratio was higher during the administration of vasopressin than during water diuresis. This has been a consistent finding in each of 12 consecutive paired experiments, as shown in Table III. The difference in the means of the urea concentration ratios during water diuresis and vasopressin infusion is highly significant statistically. As indicated in Table III, in three dogs in which the order of the experiment was reversed, the differences in the urea concentration ratios were comparable with the remainder of the group.

TABLE II

Relationship of tissue to urinary urea concentrations in Dog Ka

	Tissue urea	Urine urea	Tissue/urine conc. ratio
	μmoles/g water	*μmoles/ml*	
I. Water diuresis			
Papilla	48.0		
Inner medulla, 1	48.3	66.5	0.726
Inner medulla, 2	41.5		
II. Vasopressin and mannitol			
Papilla	56.3	60.5	0.931
Inner medulla, 1	54.7		
Inner medulla, 2	42.5		

TABLE III

Tissue to urine urea concentration ratios in 12 paired experiments

Dog	Water diuresis	Vasopressin + mannitol
	Tissue/urine urea concentration ratios	
G	0.78	0.92
H	0.48	0.62
Ka	0.73	0.93
Q*	0.62	0.91
T	0.57	0.92
V	0.62	0.71
A	0.61	0.70
C	0.68	0.95
B	0.50	0.74
E	0.86	0.92
K*	0.74	0.84
N*	0.77	0.88
Mean	0.663	0.837
SE of the mean difference		0.027
$p < 0.005$		

* Experiments in which the usual order of procedure was reversed. Vasopressin and mannitol were given initially; the second kidney was removed during water diuresis.

As measured by the clearance of inulin, unilateral nephrectomy did not appear to produce significant hemodynamic changes in the remaining kidney. As in the experiment detailed in Table I, slight changes in inulin clearance were observed during the course of single experiments. These were not consistent in magnitude or direction, however, and the mean of all inulin clearances performed during water diuresis was identical with those measured during the administration of mannitol and vasopressin.

The relatively high tissue to urine urea concentration ratios observed during the administration of vasopressin and mannitol can not be attributed solely to the presence of a mannitol diuresis. In three paired experiments, during which vasopressin was infused throughout, the effect of sodium chloride diuresis was compared with that of mannitol infusion at comparable rates of solute excretion. Kidneys removed during sodium chloride infusion showed a tissue to urine urea concentra-

tion ratio which was not different from the ratios observed during mannitol diuresis in the same animals.

These data indicate that vasopressin reduces the concentration gradient of urea between the collecting duct lumen and the medullary interstitium. Since present evidence indicates that in the dog, urea enters the medullary interstitial space (and presumably the cells of this area) by passive diffusion along a concentration gradient (13), these results suggest that vasopressin increases the permeability of the collecting duct epithelium to urea. However, an alternative explanation for these data has been considered, namely that the rate of removal of urea from the medullary interstitium is more rapid in water diuresis than during vasopressin and mannitol administration. Since a high concentration of urea within the medulla is maintained by the countercurrent exchanger effect provided by the medullary circulation, the efficiency with which urea is trapped within the medulla will vary inversely with the rate of blood flow through the capillary loops. If, during vasopressin action, either the rate of flow entering the capillary loops or the rate at which water abstracted from the collecting ducts is added to the medullary circulation is less than during water diuresis, the rate of washout of urea from the medulla would be correspondingly reduced. If either of these conditions exists, vasopressin might increase the tissue to urine concentration ratio for urea without affecting tubular permeability. It was therefore decided to determine the effect of vasopressin on the rate at which infused isotopic urea entered the medullary tissue during the steady state with respect to the urinary, and presumably medullary, urea concentration. This would serve as a measure of the turnover rate of urea in the medulla, which should be reduced if the vasopressin effect elicited in the above set of experiments were due solely to a reduction in the rate at which urea was washed out of the medullary interstitium. In addition, the effect of vasopressin on the rate of equilibration of isotopic urea between urine and renal medulla has been evaluated.

II. *The effect of vasopressin on the rate of equilibration of isotopic urea between renal cortex and medulla.* The corrected specific activities of urea in the cortex, inner medulla and papilla in kidneys removed 5 minutes after initiation of C^{14}-labeled urea infusion are shown in Table IV. Data from four paired experiments and one experiment (Dog J) in which only one kidney was removed are presented. It is apparent that in all kidneys the specific activity of the cortex is significantly higher than that of the papilla, indicating that the infused isotope was not equally distributed throughout the renal tissue. Since the infusion was continued throughout the 5 minute period, a rising plasma radioactivity was present. The gradient between cortex and papilla is attributable to a lag in the delivery of isotope to the inner medulla, whether the isotopic urea enters the medulla primarily by means of the medullary blood flow or by means of diffusion from the collecting duct. In order to assess the effect of vasopressin, the cortex to papilla gradient for C^{14}-urea has been examined. In paired experiments, this gradient was always smaller during the infusion of vasopressin than during water diuresis, indicating that vasopressin augments the rate of delivery of isotope to the renal medulla. This finding is incompatible with the possibility that the effect of vasopressin

TABLE IV

*C^{14}-urea specific activities * in kidney slices following isotope infusion*

	Water diuresis				Vasopressin and mannitol			
Dog	Cortex	Inner medulla	Papilla	Cortex/papilla	Cortex	Inner medulla	Papilla	Cortex/papilla
H	85	65	64	1.33	234	224	202	1.16
I	72	57	50	1.45	147	146	124	1.18
J	126	104	97	1.29				
K	141	103	95	1.49	359	322	290	1.24
L	123	91	101	1.22	303	295	294	1.03
			Mean	1.36			Mean	1.15

* Specific activities expressed in counts per minute per micromole of urea, corrected for quenching.

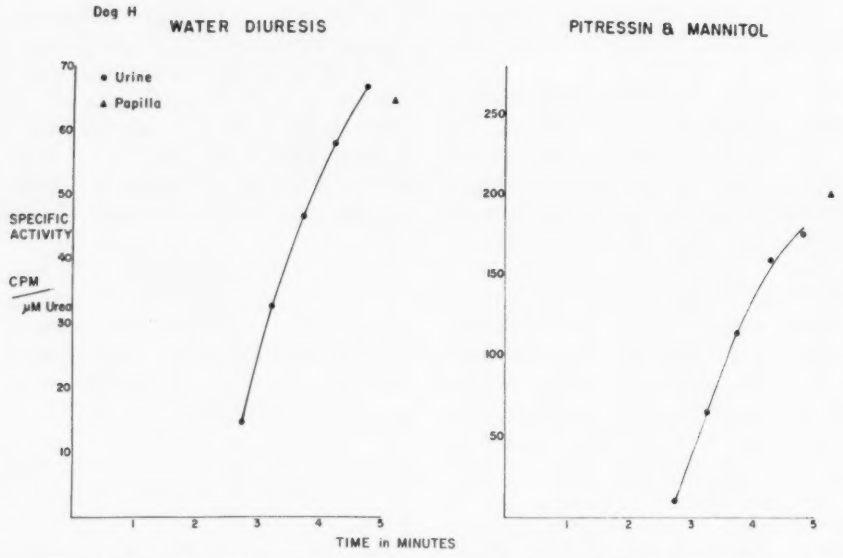

FIG. 1. THE RELATIONSHIP OF URINARY AND RENAL PAPILLARY SPECIFIC ACTIVITIES FOLLOWING C^{14}-UREA INFUSION DURING WATER DIURESIS AND DURING VASOPRESSIN ADMINISTRATION. In each part of this paired experiment, the closed circles represent urinary specific activity; triangles represent the specific activity of the papilla from the respective kidney.

on the concentration gradients of urea between tissue and urine was secondary to more efficient trapping of urea in the medulla and a diminished rate of turnover of urea in that area.

Similar qualitative results were obtained in three other paired experiments, in which lack of correction for quenching precludes absolute quantitative comparison with the studies shown in Table IV.

III. *The effect of vasopressin on the rate of equilibration of isotopic urea between urine and renal papilla.* In the experiments discussed in the previous section, urinary specific activities were also determined during the time period following the initiation of the isotopic urea infusion. In Figure 1 the specific activity of the urine in five 30-second collection periods, immediately preceding nephrectomy in a paired experiment, are plotted. In each part of the experiment, as in all other studies of this nature, the urinary specific activity was rising rapidly during this time period. The specific activity of the papilla from each kidney is also plotted. During vasopressin and mannitol infusion the specific activity of the papilla was higher than that of the final urine period. This apparent discrepancy may be attributed to the time lag between the mid-point of the final urine collection period and the removal of the kidney, since it is eliminated by extrapolation of the urinary specific activity curve to the time at which nephrectomy was performed. Thus it is suggested that during vasopressin and mannitol administration there was essentially complete equilibration of isotopic urea between urine and renal papilla 5 minutes after beginning the infusion of isotope. In contrast, during water diuresis the specific activity of the papilla was slightly lower than that of the final urine, and this difference is magnified by extrapolation of the curve of urinary specific activity. This finding suggests that movement of labeled urea from collecting duct lumen into medullary interstitium was delayed in the absence of vasopressin.

A similar relationship has been found in four other dogs, in which corrected specific activities were obtained. In Table V, the ratio of the papillary specific activity to that in the final urine has been computed in the same group of experiments shown in Table IV. In paired experiments, the tissue to urine specific activity ratio was consistently higher during the administration of vasopressin and mannitol. There is no overlap of

TABLE V

Relationship of C^{14}-urea specific activity in renal papilla and urine

	Water diuresis			Vasopressin and mannitol		
Dog	Papilla	Final urine	Papilla/ urine	Papilla	Final urine	Papilla/ urine
H	64.5	66.4	0.97	202.4	175.3	1.15
I	49.9	54.0	0.92	124.4	122.6	1.02
J	97.4	106.7	0.91			
K	94.8	113.4	0.84	289.8	264.8	1.09
L	101.3	102.5	0.99	293.8	275.5	1.07
		Mean	0.93		Mean	1.08

values when all experiments are examined; this ratio always exceeded unity during vasopressin action, and was less than 1 during water diuresis.

These data lend further support to the conclusions derived from the previously cited experiments, namely that vasopressin increases the permeability of the collecting duct epithelium to urea, and by this means promotes the movement of urea from tubular lumen to the medullary interstitial space.

DISCUSSION

The demonstration of an effect of vasopressin on the permeability of the collecting duct to urea suggests that this hormone increases the pore size in this epithelial membrane. By analogy with studies on other membranes (8, 9, 11) it may be inferred that vasopressin also augments water permeability in this segment of the nephron, and that consequently the rate of achievement of osmotic equilibrium between collecting duct tubular fluid and extratubular medullary fluid is accelerated by an action of this hormone. The maintenance of a high osmotic gradient between the medullary interstitium and collecting duct fluid during water diuresis (18, 19) can be readily explained as a result of the restricted permeability to water in the absence of vasopressin. On the basis of these observations, it is suggested that the action of vasopressin on the collecting duct epithelium constitutes an important determinant in the processes of urinary concentration and dilution.

The present method of study does not permit a kinetic analysis of the movement of urea across the collecting duct epithelium. Whether vasopressin augments the diffusion of urea, or whether the increased rate of movement of urea is primarily a result of solvent drag imposed by the osmotic flow of water can not be determined. In either event, augmentation of urea accumulation in the medulla by the action of vasopressin serves to increase the degree to which the kidney can conserve water and concentrate the urine.

A hypothesis concerning the mechanism whereby urea contributes to the process of urinary concentration has been previously presented by Berliner, Levinsky, Davidson and Eden (18). The results of the present study are compatible with their formulation, and serve to emphasize that the unique role of urea is dependent upon special permeability characteristics of the collecting duct epithelium with respect to this molecule. This is illustrated by the following considerations. Complete impermeability to urea would preclude the movement of urea into the medullary interstitial space, and limit the solute concentration attainable in that space to the concentration of sodium chloride which can be achieved by active transport. This latter concentration would consequently determine the limit of maximal urinary osmolality. If the collecting duct offered no barrier to the diffusion of urea, the movement of water out of the tubule would be accompanied by urea at a concentration identical with that within the tubular fluid. Consequently, the urea concentration within the lumen would remain uniform along the length of the collecting duct. As a result, although urea would be added to the interstitial fluid solute, its contribution would be limited to that concentration of urea present in the fluid leaving the distal convolution. Clearly, the degree of permeability to urea in the collecting duct lies between these two extremes. There exists a sufficient barrier to diffusion to permit a rising concentration within the lumen as water is abstracted, yet movement of urea into the medullary interstitium is sufficient to add materially to the total solute concentration within the medulla.

Concentration of urea within the tubular fluid as it passes down the collecting duct is dependent upon osmotic abstraction of water by nonpermeating solute (sodium chloride) in the peritubular space. Osmotic movement of water will proceed until the interstitial sodium chloride is balanced by an osmotically equivalent concentration of nonpermeating solute within the tubular lumen. Urea, since it can penetrate the tubular epithelium, will exert little effective restriction to the continued

movement of water out of the collecting duct. Consequently, the ratio of urea to nondiffusible solute in the fluid entering the collecting ducts from the distal convolution will determine in part the fraction of that fluid which can be abstracted from the tubule, and thus the extent to which urea can be concentrated within the tubular and interstitial fluids and ultimately contribute to urinary concentration. Thus, at comparable rates of solute excretion, urine osmolality is significantly higher during urea osmotic diuresis than when sodium chloride or mannitol is used as the loading solute (*20*, *21*). In contrast, when urea constitutes a small fraction of the total solute entering the collecting duct, as in a subject fed a low protein diet, the contribution of urea to urinary concentration is minimized, and maximal concentrating ability is impaired (*13*, *22*).

During dehydration, urea clearance—relative to filtration rate—diminishes (*23*), and the plasma urea concentration accordingly rises. It is suggested that these accompaniments of the dehydrated state are manifestations of the role of urea in renal water conservation. In the hydropenic animal elaborating a highly concentrated urine, the urea concentration within the renal medulla exceeds the total solute concentration in the plasma. In the dog, concentrations of 700 to 800 μmoles of urea per g of water are frequently observed in the renal papilla (*13*). Barring an active transport process for urea, its concentration in the medulla can not exceed the net concentration of urea in the water entering the medullary interstitium from the collecting duct. Consequently, it may be concluded that in the hydropenic dog producing a highly concentrated urine, the fluid entering the papillary interstitium from the collecting duct contains sufficient urea so that it is hypertonic to the systemic body fluids. Since this process occurs physiologically only in the hydropenic state, it may be viewed as a homeostatic mechanism which acts to conserve body water at the expense of increasing body fluid osmolality. Since urea diffuses readily into the various body fluid compartments, the major need of the organism, maximal conservation of water, is subserved without incurring potentially detrimental fluid shifts between these compartments.

Recent studies indicate that animals residing in arid regions possess an unusually deep renal medulla and papilla (*24*), in contrast to certain species whose habitat is restricted to areas in which the water supply is abundant (*24*, *25*). In addition to increasing the efficiency of the countercurrent mechanisms, this anatomical characteristic favors the achievement of the maximal attainable intratubular urea concentration and similarly increases the degree to which urea will attain diffusive equilibrium between tubular and peritubular fluids. These observations suggest that the role of urea in urinary concentration is vital to the maintenance of body fluid volume in the normal existence of certain animal species.

SUMMARY

The effect of vasopressin on the relative concentrations of urea within the renal medulla and within the urine has been determined in a set of paired steady state experiments in dogs. In every experiment a higher tissue to urine concentration ratio for urea has been observed during vasopressin administration than during water diuresis. In additional studies in which C^{14}-labeled urea was injected intravenously it was observed that the rate of appearance of isotopic urea in the renal medullary tissue is enhanced by vasopressin, suggesting that vasopressin increases the rate of turnover of urea within the renal medulla. Similarly, equilibration of labeled urea between urine and renal papilla was more rapid in the presence of vasopressin. These data indicate that the rate of movement of urea from the collecting duct lumen to the peritubular fluid is augmented by the action of vasopressin. Since this evidence favors the view that passage of urea out of the tubular fluid in the dog kidney is by means of passive diffusion along a concentration gradient, the present results are interpreted to indicate a direct effect of vasopressin on the permeability of the collecting duct epithelium.

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EFFECT OF STEROIDS ON WATER DIURESIS AND VASOPRESSIN SENSITIVITY *

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The ability of glucocorticoids to reverse the abnormal diuretic response to water in adrenal insufficiency (1–5) and to increase water diuresis in normal subjects (6, 7) is well known. Although glucocorticoids appear to be specific in this regard, the possibility that other steroids may have a pharmacologic effect on water diuresis in normal man has not been investigated systematically. The present study was therefore undertaken to compare the effects of large doses of cortisol, progesterone, estrogen and aldosterone on the maximal rate of free water clearance during water diuresis in normal man. The effects of low sodium diet and an aldosterone antagonist also were examined. It was found that only cortisol appeared to have a specific effect on this rate. Variations in maximal water diuresis also were encountered in control studies and after treatment other than cortisol, but these appeared to be related to changes in total solute excretion.

It has been suggested that glucocorticoids may antagonize the action of antidiuretic hormone (1, 6, 8). To test this possibility, the effect of a constant infusion of a submaximal dose of exogenous vasopressin on water diuresis was examined before and after treatment with cortisol and other steroids. Variations in the response to vasopressin were encountered which were related to variations in the initial maximal rate of water diuresis in control studies. None of the agents tested, including cortisol, appeared to alter this relationship.

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METHODS

Five healthy, paid volunteer medical students served as subjects. The experiments were performed in the postabsorptive state, but at different times of day. After an initial oral water load of 20 ml per kg of body weight, the subject maintained water diuresis by drinking an amount of water equal to the amount voided during the last urine collection period. Urine collections were obtained by voluntary voiding at 15-minute intervals. An infusion of 5 per cent dextrose in water was maintained at a constant rate of 0.6 or 1.2 ml per minute throughout the experiment. Blood samples for determination of total solute, electrolyte and creatinine concentration were obtained at the beginning and the end of the infusion. In order to minimize antidiuretic stimuli, no injection or venipuncture was performed during the experiment except for 3 experiments in Subject L.B., in which inulin clearance was measured. After a steady rate of water diuresis had been established for at least 3 periods, the infusion was changed to one containing vasopressin in an amount to provide 4, 8, 12 or 16 mU per hour. A freshly opened ampule of commercial vasopressin (Pitressin; Parke, Davis & Co.) was used for each experiment. One lot (S216F) was used for 4 subjects. The lot used in Subject L.B. (Y108HA) had less antidiuretic potency when the two lots were compared in the same experiment. In most experiments, the response to a single rate of vasopressin administration was observed. In selected experiments, two different rates were used. Each rate was continued for 90 to 120 minutes, until the urine flow was again steady. After vasopressin was discontinued, the return to a new steady state of maximal water diuresis was observed. This usually required 75 to 90 minutes. The data from such experiments are presented as the mean values for 2 to 4 consecutive periods in which urine flow did not vary by more than 10 per cent during maximal water diuresis, or by more than 0.5 ml per minute during vasopressin infusion.

In addition to 3 or 4 control experiments in each subject, the experiments were repeated after the following forms of treatment.

1. Cortisol. Three subjects took 40 mg of cortisol orally every 6 hours for 4 days.

2. Progesterone. Two subjects received intramuscular injections of 100 mg of progesterone in oil every 12 hours for 4 days.

3. Estrogen. Two subjects took 0.1 mg of ethinyl estradiol orally every 6 hours for 7 days.

4. Aldosterone. Three subjects received intramuscular injections of 0.3 mg of *dl*-aldosterone monoacetate every 6 hours for 3 days. A fourth subject (R.B.) was studied after 1 day of aldosterone administration.

5. Low salt diet. Three subjects ingested a 200 mg sodium diet for 3 to 5 days prior to study.

6. Spironolactone. At the completion of the experiment on a low sodium diet, these subjects took 200 mg of spironolactone, an aldosterone antagonist, orally every 6 hours for 2 days while remaining on a low sodium intake. A fourth subject (A.G.) was studied after receiving spironolactone for 4 days while on an unrestricted diet.

Chemical methods and calculations

Methods for determination of osmolality, creatinine, sodium and potassium have been described previously from this laboratory (9). The net excess of water in the urine, or free water clearance (C_{H_2O}), was calculated as the difference between urine flow and osmolal clearance. Creatinine clearance values served as a check on the constancy of bladder emptying and as a rough measure of glomerular filtration rate. Inulin clearance (10) was measured in three experiments in Subject L.B. Antidiuresis occurred with blood sampling and at the time of injection of the inulin prime, and the experiments had to be prolonged in order to obtain data on steady state water diuresis. For this reason, inulin clearance was not measured in any other experiments. The response to vasopressin was measured as the change in free water clearance which occurred between the initial maximal water diuresis and the steady state during vasopressin infusion. This has been termed ΔC_{H_2O} (11).

RESULTS

The results are summarized in Table I.

1. Maximal water diuresis. Values for free water clearance during maximal water diuresis varied considerably. In control studies, changes in maximal C_{H_2O} could be related to variations in total solute excretion (Figure 1). This relationship was observed whether the variation in solute excretion could be related to diurnal rhythm or to a change in dietary intake. Although sodium excretion and total solute excretion often were parallel, the former showed a greater variability, and the correlation between sodium excretion and C_{H_2O} was not significant in control experiments. Data obtained after progesterone and estrogen administration showed slightly high values for sodium excretion, total solute excretion and C_{H_2O}. After intramuscular administration of aldosterone, sodium excretion was somewhat decreased compared with the mean control values, and potassium excretion was increased. However, total solute excretion and C_{H_2O} decreased only in Subject L.B. A marked decrease in sodium excretion and a de-

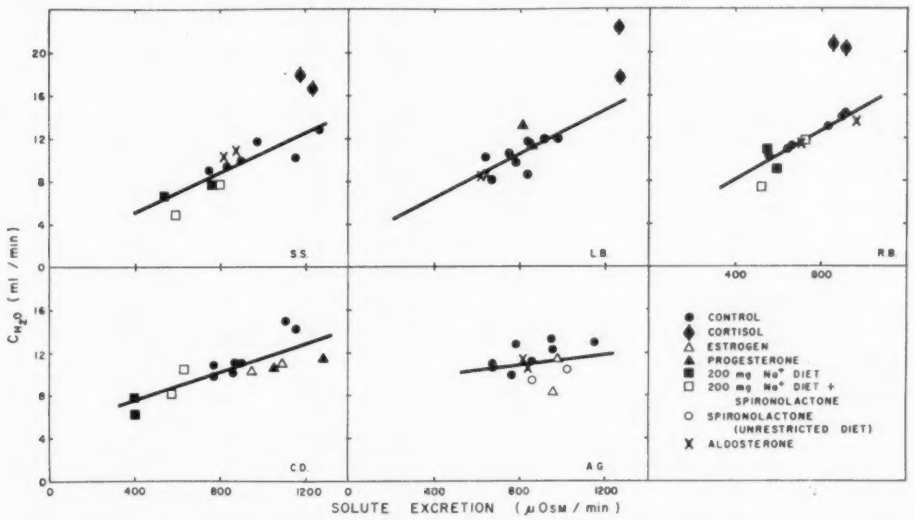

FIG. 1. MAXIMAL FREE WATER CLEARANCE (C_{H_2O}) VERSUS TOTAL SOLUTE EXCRETION. Data are shown for the subjects individually before and after the administration of cortisol, estrogen, progesterone, aldosterone, a low sodium diet and an aldosterone antagonist. The solid lines represent the regression line calculated by the method of least squares for all data for each subject exclusive of values obtained after cortisol administration.

TABLE I

Water diuresis and vasopressin response in normal young men. Control studies and effect of cortisol (Cort), progesterone (Prog), estrogen (Estr), aldosterone (Ald), 200 mg sodium diet (Low Na) and spironolactone on low sodium diet (Sp + low Na) or on unrestricted diet (Sp) are shown. Time given is for the beginning of vasopressin infusion

Subject Age Surface Area	Study Date Time	Vaso-pressin mu/hr	Urine Flow ml/min	Urine Osmolality mOsm/L	Solute Excretion μOsm/min	CH_2O ml/min	Sodium Excretion μEq/min	Creatinine Clearance ml/min	ΔCH_2O ml/min
S. S.	Control	0	15.0	65	974	11.6	126	104	
24	10/16/59	4	6.8	158	1071	3.0	194	99	8.6
1.98m^2	9:30 am	0	13.1	68	896	9.9	130	100	
	Cort	0	20.5	60	1230	16.3	240	146	
	10/23/59	4	8.5	98	837	5.6	71	120	10.7
	9:45 am	8	2.8	312	874	-.3	120	140	16.6
		0	21.8	53	1155	17.7	292	155	
	Control	0	16.8	76	1265	12.3	189	123	
	11/2/59	4	4.5	190	849	1.5	190	121	10.8
	9:45 am	8	1.7	469	769	-1.1	234	119	13.4
		0	14.0	78	1102	10.1	305	112	
	Low Na	0	10.4	73	760	7.7	7	115	
	11/6/59	4	3.1	167	512	1.2	12	104	6.5
	10:15 am	0	8.5	64	538	6.5	41	107	
	Sp+low Na	0	10.5	76	798	7.7	110	112	
	11/9/59	4	2.4	254	613	.2	78	103	7.5
	10:30 am	0	6.8	87	596	4.7	70		
	Control	0	11.6	64	748	8.9	43	104	
	2/27/60	4	4.1	164	675	1.7	128	117	7.2
	12:45 pm	0	12.3	68	828	9.3	146	119	
	Ald	0	14.1	58	817	11.2	76	122	
	3/5/59	4	4.9	151	744	2.3	84	113	8.9
	1:30 pm	0	13.9	62	870	10.8	158	119	
C. D.	Control	0	18.8	58	1100	14.9	120	155	
24	7/22/59	8	5.4	168	895	2.1	115	142	12.8
2.03m^2	9:45 am	0	12.6	61	769	9.8	160	191	
	Prog	0	16.0	79	1280	11.5	201	144	
	7/29/59	8	4.4	238	1060	0.6	230	128	10.9
	10:00 am	0	14.3	73	1055	10.5	175	127	
	Control	0	14.2	63	896	11.0	72	122	
	10/2/59	8	5.0	172	860	1.9	152	144	9.1
	2:30 pm	0	14.3	62	870	11.1	137	138	
	Estr	0	14.8	73	1083	10.9	188	138	
	10/9/59	8	5.5	205	1121	1.4	282	161	9.5
	1:30 pm	0	13.7	69	944	10.3	202	145	
	Control	0	18.3	63	1147	14.1	161	102	
	11/18/59	8	5.2	150	772	2.3	99	112	11.8
	10:30 am	0	13.6	54	718	10.8	106	123	
	Low Na	0	7.8	52	404	6.3	9	148	
	11/23/59	8	0.7	479	356	-.6	2	152	6.9
	1:30 pm	0	9.3	44	404	7.8	12	126	
	Sp+low Na	0	12.8	50	635	10.4	41	128	
	11/25/59	8	3.4	193	652	0.9	65	132	9.5
	10:00 am	0	10.3	56	578	8.1	40	87	
R. B.	Control	0	13.6	49	665	11.2	43	89	
24	11/4/59	4	8.6	90	774	5.8	97	101	5.4
1.80m^2	9:45 am	8	2.0	348	696	-.5	124	99	11.7
		0	17.2	52	895	14.0	172	102	
	Cort	0	23.5	39	916	20.2	91	105	
	11/14/59	4	14.6	56	817	11.6	92	101	8.6
	12:45 pm	8	6.6	121	802	3.6	112	78	16.6
		0	23.8	36	856	20.6	169	110	
	Control	0	12.4	45	558	10.3	25	78	
	11/27/59	4	7.4	92	677	4.8	101	83	5.5
	11:15 am	0	15.6	42	646	13.0	70		
	Low Na	0	11.3	54	597	9.1	4	97	
	11/30/59	4	4.7	122	572	2.6	7	95	6.5
	1:30 pm	0	13.0	42	546	10.9	8		

Table I. (cont.)

Subject Age Surface Area	Study Date Time	Vaso-pressin mu/hr	Urine Flow ml/min	Urine Osmolality mOsm/L	Solute Excretion μOsm/min	CH_2O ml/min	Sodium Excretion μEq/min	Creatinine Clearance ml/min	ΔCH_2O ml/min
R. B. (cont.)	Sp+low Na	0	9.2	57	525	7.3	19	99	
	12/2/59	4	4.5	117	531	2.5	35	94	4.8
	10:30 am	0	14.2	50	704	11.6	83	98	
	Control	0	15.9	52	832	12.9	77	105	
	12/19/59	4	10.2	92	950	6.8	196	100	6.1
	10:30 am	0	17.4	52	907	14.1	305	110	
	Ald	0	14.0	50	706	11.5	38	103	
	12/28/59	4	8.7	105	911	5.4	116	105	6.1
	10:30 am	0	16.9	57	965	13.4	155	102	
A. G.	Control	0	16.9	68	1150	12.9	286	146	
25	4/17/59	8	3.3	240	800	.5	162	127	12.4
1.80m^2	10:45 am	0	15.6	50	782	12.8	142	172	
	Control	0	15.6	61	959	12.2	95	144	
	9/11/59	8	2.5	286	720	-.1	140	149	12.3
	10:00 am	0	14.2	61	862	11.1	164	172	
	Estr	0	14.7	66	978	11.3	126	208	
	9/20/59	8	4.1	290	1178	-.2	210	181	11.5
	10:45 am	0	11.7	82	950	8.2	178	142	
	Control	0	13.2	51	675	10.8	72	171	
	10/5/59	8	2.4	268	623	.1	116	150	10.7
	10:45 am	0	16.7	56	951	13.2	225	179	
	Sp	0	14.1	72	1021	10.4	184	148	
	10/10/59	8	3.0	316	947	-.4	200	142	10.8
	11:00 am	0	12.4	70	860	9.3	155	154	
	Control	0	12.5	62	765	9.8	68		
	11/16/59	8	1.1	551	584	-1.1	55		10.9
	2:00 pm	0	13.2	51	673	10.7	140		
	Ald	0	13.8	60	818	10.8	103		
	3/5/60	8	2.8	300	830	-.2	106		11.0
	10:30 am	0	13.6	62	842	10.6	103		
L. B.	Control	0	14.7	56	830	11.7	109	92	
26	12/31/59	8	12.4	67	830	9.4	120	115	2.3
2.00m^2	1:45 pm								
	Control	0	10.5	64	666	8.1	33	95	
	1/8/60	16	1.9	281	525	.0	77	100	8.1
	1:45 pm	0	12.6	50	635	10.2	105	105	
	Prog	0	14.6	58	842	11.6	104	122	
	1/15/60	16	4.1	169	687	1.6	63	107	10.0
	1:15 pm	8	9.6	78	744	6.8	112	110	4.8
		0	16.1	50	807	13.1	161	114	
	Control	0	11.4	73	833	8.5	89	134*	
	1/22/60	16	3.4	211	694	0.9	94	120	7.6
	2:00 pm	0	13.2	56	746	10.6	80	143	
	Ald	0	10.4	59	615	8.2	38	134*	
	1/29/60	12	4.1	153	621	1.9	70	135	6.3
	2:45 pm	0	10.8	59	634	8.6	79	128	
	Control	0	15.1	60	908	11.9	75	132	
	2/5/60	12	6.9	128	880	3.7	111	110	8.2
	10:00 am	8	9.2	89	821	6.3	111	106	5.6
		0	12.6	61	778	9.8	61	126	
	Cort	0	22.4	56	1265	17.8	336	130*	
	4/9/60	16	9.9	124	1236	5.5	215	125	16.9†
	12:00 am	8	22.6	63	1418	17.6	318	132	4.8†
		0	26.8	47	1261	22.4	328	136	

*Mean inulin clearance values were 109 ml. per minute for Control experiment, 115 ml. per minute after Aldosterone and 160 ml. per minute after Cortisol.

†Water diuresis was increasing when vasopressin infusion begun, therefore, ΔCH_2O was measured from final water diuresis in this experiment.

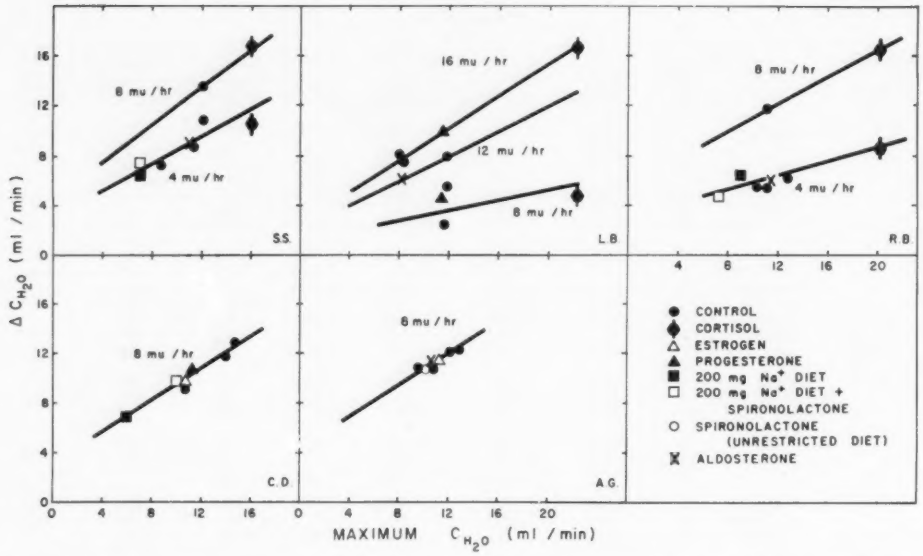

FIG. 2. VASOPRESSIN RESPONSE (ΔC_{H_2O}) VERSUS MAXIMAL FREE WATER CLEARANCE (MAXIMAL C_{H_2O}). Data are shown for subjects individually before and after the administration of cortisol, estrogen, progesterone, aldosterone, a low sodium diet and an aldosterone antagonist. The regression lines shown were drawn visually to represent the equation $\Delta C_{H_2O} = k$ (max. $C_{H_2O} + a$). The intercept on the abscissa (value of constant a) was the same at two different dose levels in Subjects S.S., L.B. and R.B. The response to 8 mU per hour of a less potent vasopressin preparation in L.B. was less consistent.

crease in total solute excretion and C_{H_2O} was observed on a low salt diet. When spironolactone was administered to these subjects, sodium excretion increased strikingly, but this was accompanied by an increase in C_{H_2O} and total solute excretion only in Subject C.D. In all of the foregoing experiments, the relationship between total solute excretion and C_{H_2O} was similar to that observed in control studies. After cortisol administration, there was a variable increase in sodium and total solute excretion; however, C_{H_2O} increased markedly in each subject out of proportion to the change in solute excretion.

Creatinine clearance values could not be related to changes in C_{H_2O} in the present study. Subject S.S. showed a rise in creatinine clearance on cortisol. In Subject L.B., inulin clearance increased on cortisol but creatinine clearance did not. Creatinine clearance was not altered consistently by any of the other treatments studied.

II. Vasopressin sensitivity. The response to a given dose of vasopressin, in terms of its effect on free water clearance (ΔC_{H_2O}), was not constant in the same subject (Figure 2). As the value of C_{H_2O} obtained during initial maximal water diuresis increased, the value for ΔC_{H_2O} obtained in the steady state by the infusion of a given dose of vasopressin also increased. This relationship can be described by the equation:

$$\Delta C_{H_2O} = k \text{ (maximal } C_{H_2O} + a)$$

where k is a constant which varies with the dose of vasopressin and a is a constant amounting to 4 to 10 ml per minute in different subjects. For the few data available, the value of a appeared to be the same at different dose levels in the same subject. This relationship between ΔC_{H_2O} and maximal C_{H_2O} accounts for much of the variability encountered both in control studies and after treatment. Thus, the increase in maximal C_{H_2O} after cortisol administration is accompanied by an increase in ΔC_{H_2O}, particularly in response to the infusion of larger doses of vasopressin.

DISCUSSION

Variations in the diuretic response to a water load which cannot be attributed to changes in endogenous antidiuretic hormone production have been described previously. These have been related to glomerular filtration rate (12), solute

excretion (12, 13) and sodium excretion (13, 14). In the present study, a close relationship between maximal C_{H_2O} and total solute excretion was observed in normal subjects with diurnal, dietary and hormonally induced variations in solute excretion. It is possible that under these circumstances variations in both C_{H_2O} and total solute excretion are determined by the amount of sodium and water delivered to the distal diluting site. Subsequent variations in reabsorption and exchange of sodium in the distal tubule could account for the lack of correlation between urinary sodium and C_{H_2O}. In osmotic diuresis delivery of non-reabsorbable solute to the distal tubule can result in an increase in solute excretion without an associated increase in C_{H_2O} (14–16).

As previously noted (7), cortisol increases C_{H_2O} out of proportion to its effect on total solute excretion. This increase has been attributed to the effect of an increase in glomerular filtration rate, together with either a redistribution of solute reabsorption between proximal and distal tubules, or an alteration in tubular permeability to water (5, 7).

A few observations are available which suggest that maximal water diuresis is increased in pregnancy (17, 18). Progesterone administration has been reported to enhance the rate of water elimination in rats (19) while estrogen has no effect (6). In the present study, progesterone and estrogen had little effect on water diuresis in normal young men. Moreover, in preliminary experiments, water diuresis showed no consistent variation at different phases of the menstrual cycle in normal young women (20).

Conceivably, aldosterone could alter the relationship between C_{H_2O} and solute excretion by enhancing direct sodium reabsorption at sites where free water is made available. In the present study, such an effect could not be detected with exogenous aldosterone or when endogenous aldosterone production was increased by sodium restriction. Similarly, an aldosterone antagonist failed to decrease free water clearance. However, the variability of water diuresis in these chronic experiments may have masked slight alterations in C_{H_2O} caused by aldosterone.

It is possible that small changes in glomerular filtration rate could account for the changes in C_{H_2O} and total solute excretion observed in the present study. Creatinine clearance measurements could not detect such changes, particularly where only initial and final blood values were measured. Unfortunately, we have been unable to obtain inulin clearance measurements without interfering with maximal water diuresis in our subjects.

In the present study, ΔC_{H_2O} in response to a given dose of vasopressin was found to vary with the initial value of maximal C_{H_2O}. Hollander, Williams, Fordham and Welt (11) also noted an increase in ΔC_{H_2O} in response to the same dose of vasopressin in experiments in which solute excretion was increased and initial maximal C_{H_2O} was high. Theoretically this relationship would be expected if the action of vasopressin were to increase the permeability of the cell membrane (21), since the amount of water reabsorbed would then depend upon the mean osmotic gradient along the distal tubule during vasopressin administration. When more free water was available in the distal tubule, the amount reabsorbed in response to vasopressin was greater, but the final urine was also more dilute. Both the lower urine osmolality and the larger value of ΔC_{H_2O} would appear to be the physicochemical consequence of the change in available free water and do not indicate a specific change in the sensitivity of the renal tubules to vasopressin. On the other hand, more endogenous antidiuretic hormone secretion may be required to concentrate the urine and maintain the water economy under conditions when maximal C_{H_2O} is increased. This change in requirement would apply whether C_{H_2O} was altered by cortisol or by any other means (15).

Whatever its theroetical basis, the observed relationship between ΔC_{H_2O} and maximal C_{H_2O} can be used to assess variations in vasopressin response under experimental conditions. Previous studies on the effect of glucocorticoids on vasopressin response based on the effect of a single injection (7) indicated that the tubule was not insensitive to the action of ADH. Using the more precise technique of the present study, neither cortisol nor any of the other steroids tested altered the sensitivity of the renal tubule to vasopressin.

SUMMARY

The maximal rate of water diuresis and the response to a constant infusion of vasopressin were

studied before and after the administration of cortisol, progesterone, estrogen, aldosterone, a low sodium diet and an aldosterone antagonist in healthy young men.

Variations in free water clearance during maximal water diuresis were observed. These variations could be related to changes in total solute excretion in control experiments. This relationship was not altered by any of the treatments except cortisol administration. After cortisol administration, free water clearance increased more than would be expected from the change in solute excretion.

The decrease in free water clearance observed with the infusion of a given submaximal dose of vasopressin was directly related to the initial maximal rate of free water clearance. This relationship was unchanged by treatment, including cortisol administration.

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LEUKOKINETIC STUDIES. III. THE DISTRIBUTION OF GRANULOCYTES IN THE BLOOD OF NORMAL SUBJECTS *

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In a previous publication (1) it was noted that when granulocytes were labeled *in vitro* with radioactive diisopropylfluorophosphate (DFP^{32}) and then returned to the circulation of the donor, about half of the labeled cells could not be found in the circulation at the completion of the infusion (T_0). Thereafter the remaining labeled cells left the circulation in a random fashion with a mean half-time disappearance ($T_{\frac{1}{2}}$) of 6.6 hours.

Since cell damage and significant elution of the label could not be demonstrated under the conditions of the study, it was suggested that the immediate disappearance of half the infused cells was due to their rapid dilution in a larger pool than that calculated from the blood volume and the venous granulocyte count.

The concept that the circulating granulocyte pool (CGP) does not constitute all of the intravascular leukocytes is not new. Vejlens (2) reviewed the numerous investigations carried out up to 1938 and pointed out that as early as 1867 Cohnheim observed white cells in a marginal position along the walls of venules. By microscopic examination it has since been demonstrated that leukocytes may circulate freely in the blood, adhere to the vascular endothelium in sites where the blood flow is relatively slow and then once again re-enter the circulation in a process of continuous exchange (2).

If DFP^{32}-labeled granulocytes come into rapid equilibrium with marginated granulocytes, it might be expected that one could account for most if not all of the labeled cells by mobilizing the marginated cells into the circulation with epinephrine. Mobilization of the marginated cells into the circulation after equilibration has occurred should not alter the specific activity of the cells in the CGP.

The purpose of this paper is to present evidence that labeled granulocytes are distributed in a larger pool than the CGP and to present data on the size of this distribution compartment in 45 normal subjects.

MATERIAL AND METHODS

Volunteers from the Utah State Prison were used in these studies. All of the subjects were healthy cooperative males, 20 to 50 years of age, with normal leukocyte values, who were free of even minor infections. The subjects were allowed to have breakfast at 6 a.m. The infusion of labeled blood was begun between 8 and 10 a.m.

The methods for labeling granulocytes *in vitro* (1), for the isolation of leukocytes from the blood samples and for the determination of leukocyte radioactivity were described previously (3).

Total and differential leukocyte counts were made at the beginning of an experiment and at appropriate time intervals during the study. When reference is made in the text to granulocyte pools, these terms refer to the neutrophilic granulocyte series. Eosinophils and basophils have not been included in the granulocyte total because, by means of autoradiographic studies with tritiated DFP, it has been found that these cells do not label (4).

The number of granulocytes per millimeter cubed was calculated from the total and the differential leukocyte counts. The blood volume of a subject was assumed to be 2.68 L per m^2 of body surface area (5). The surface area was estimated from the nomogram of DuBois (6). The circulating granulocyte pool was calculated from the number of granulocytes per millimeter cubed and the estimated blood volume.

Three 20 ml samples of blood were removed from the bag just prior to the infusion, and the specific activity of the isolated leukocytes, expressed as counts per minute per milligram of leukocyte nitrogen, was determined. The mean value for the 3 determinations was taken to represent the specific activity of the granulocytes in the bag (SA of bag G). The concentration of granulocytes in the bag was calculated from the total and differential leukocyte counts determined directly on the bag blood after incubation with DFP^{32}. The volume of blood ad-

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ministered to the subject, usually between 450 and 500 ml, was calculated from the weight of blood injected and an assumed specific gravity of 1.055. The total number of labeled granulocytes given to the subject (no. G in bag) was then calculated.

The blood was administered to the original donor over a 15 to 20 minute period. Within 3 minutes after the completion of the infusion (T_0), three 20 ml samples of blood were withdrawn from the subject and the specific activity of the granulocytes in the circulation was determined. The mean value of the 3 determinations was taken to represent the specific activity of the granulocytes in the blood at T_0 (SA blood G).

The percentage of infused cells present in the CGP at T_0 was calculated as follows:

$$\text{Per cent of infused G present in CGP at } T_0 = \frac{\text{CGP} \times \text{SA blood G}}{\text{no. G in bag} \times \text{SA of bag G}} \times 100.$$

The number of cells in the total blood granulocyte pool (TBGP) was calculated as follows:

$$\text{TBGP} = \frac{\text{SA of bag G} \times \text{no. G in bag}}{\text{SA of blood G}}.$$

RESULTS

Influence of epinephrine and exercise on the percentage of infused granulocytes present in the circulation at T_0. In an attempt to account for the labeled cells apparently lost from the circulation during the infusion, the following studies were carried out. Blood from each of five subjects was labeled with DFP^{32}, infused into the donor, and the percentage of infused graulocytes present in the circulation at T_0 determined. Five days later the studies were repeated in the same subjects except that 0.5 mg of epinephrine was added to the labeled blood just prior to infusion. In each epinephrine study, as compared with the control, there was an increase in granulocyte count at the end of the infusion ranging from 37 to 179 per cent. In addition, the percentage of infused granulocytes in the circulation increased from a mean of 48.0 to a mean of 78.8. Nevertheless, in no instance could all of the infused cells be accounted for in the circulation (Table I).

Similar studies were carried out in four additional subjects except that a 440 yard run at the completion of the infusion was used to mobilize granulocytes rather than epinephrine. After exercise the granulocyte count increased 64 per cent on the average and the proportion of infused granulocytes which could be accounted for in the circulation increased from an average of 45 to 79 per cent. The results varied considerably. It is of interest that in two subjects (III-4 and III-10) 96 and 100 per cent of the infused cells were found in the circulation (Table II).

TABLE I

Influence of epinephrine on the percentage of infused granulocytes present in the CGP at T_0 *

Subject	Per cent infused G in CGP at T_0		Δ G†
	Control	Epinephrine	
II-164	47	88	1.72
II-166	55	82	1.37
II-168	52	81	1.42
II-204	49	71	1.99
II-208	37	72	2.79
Mean	48.0	78.8	1.86

* G = granulocytes; CGP = circulating granulocyte pool; T_0 = completion of infusion.
† Ratio of granulocyte count at T_0 of epinephrine study to that in the control study.

Influence of epinephrine and of exercise on the specific activity of the granulocytes in the circulation. To assess the possibility of a continual exchange of granulocytes between the CGP and the marginated cells, each of eight subjects was given an infusion of autogenous, DFP^{32}-labeled blood. Three hours after completion of the infusion, at which time the granulocyte disappearance curve was well established, the concentration and the specific activity of the granulocytes in the CGP were determined. Immediately thereafter 0.4 mg epinephrine in 50 ml of isotonic saline was administered intravenously over a period of 4 to 27 minutes. The concentration and the specific activity of the granulocytes were measured again. In two subjects the granulocyte count increased

TABLE II

Influence of exercise on the percentage of infused granulocytes present in the CGP at T_0

Subject	Per cent infused G in CGP at T_0		Δ G*
	Control	Exercise	
III-2	30	54	1.73
III-4	43	101	2.38
III-10	62	96	1.25
III-12	55	65	1.18
Mean	45.0	79.0	1.64

* Ratio of granulocyte count at T_0 of exercise study to that in the control study.

TABLE III

The effect of epinephrine and exercise on granulocyte specific activity (SA) *

	SA, cpm per mg N		
	Before	After	Δ G†
Epinephrine infusion‡	1,387	1,331	1.47
Exercise (440 yard run)§	697	756	1.23

* The epinephrine or exercise was administered 3 hours after infusion of the labeled blood.
† Ratio of granulocyte count after epinephrine injection or exercise to that before.
‡ Values are the mean of 6 subjects.
§ Values are the mean of 2 subjects.

less than 10 per cent and these studies were, therefore, discarded. In the other six subjects, the mean granulocyte count increased 47 per cent and yet there was no significant change in the specific activity of the granulocytes in the CGP (Table III).

A similar study was carried out in three subjects except that a 440 yard run was used in place of epinephrine. Neutrophilia was not observed in one subject. Therefore, this subject is not included in Table III. The mean granulocyte count in the other two subjects increased 23 per cent in about 2 minutes with no significant change in the specific activity of the granulocytes in the CGP.

Inability of epinephrine to mobilize damaged granulocytes. To determine whether cells which have been deliberately damaged and presumably have been rapidly removed from the CGP could be mobilized back into the CGP by an infusion of epinephrine, blood was withdrawn from four subjects, labeled with DFP^{32} and stored for 24 hours at 4° C. Storage of blood has previously been shown to result in the immediate disappearance from the CGP of over 90 per cent of the injected cells (1). The cells were then infused into the respective donors and the fraction of the injected cells present in the circulation at T_0 was determined. No untoward reactions occurred during or after the infusions and the granulocyte counts remained unchanged.

TABLE IV

Failure of epinephrine to mobilize damaged granulocytes

Subject	Per cent of infused G present in CGP at T_0	
	Stored cells	Stored cells + epinephrine
I-119	9.3	9.9
I-121	5.3	5.9
I-153	8.0	6.8
I-155	3.9	5.4
Mean	6.6	7.0

The studies were repeated in the same individuals at a later date, and 0.4 mg of epinephrine was added to the blood in the bag. The results are presented in Table IV. The damaged granulocytes were rapidly removed from the circulation during the infusion and were not in the circulation at T_0. Furthermore, the epinephrine failed to mobilize the damaged cells even though a mean increase in granulocyte count of 23 per cent occurred.

The size of the TBGP and CGP in 45 normal subjects. Since the apparent loss of 50 per cent of the DFP^{32}-labeled granulocytes during the infusion appears to be due to their distribution in a pool larger than the CGP and not due to cell damage, the size of this distribution compartment (TBGP) was measured in 45 normal subjects (Table V).

The mean TBGP was 65.3×10^7 granulocytes per kg of body weight and the mean CGP was 30.7×10^7 granulocytes per kg.

Influence of epinephrine and of exercise on the distribution of granulocytes within TBGP. To ascertain the effect of epinephrine on the size of the TBGP and the distribution of cells therein, blood from each of eight subjects was labeled with DFP^{32}, infused into the donor, and the TBGP and CGP were determined. Five days later the studies were repeated in the same subjects except that 0.5 mg epinephrine was added to the labeled blood. The size of the TBGP was unaffected by the epinephrine (Table VI). However, the mean number of granulocytes in the CGP increased from 34.2×10^7 cells per kg to 50.5×10^7 cells per kg, a 48 per cent increase after administration of epinephrine.

TABLE V

The size of the total blood granulocyte pool (TBGP) and circulating granulocyte pool (CGP) in 45 normal subjects

	Mean ±1 SD	Range
TBGP, cells $\times 10^7$/kg	65.3 ± 22.7	36–117
CGP, cells $\times 10^7$/kg	30.7 ± 11.8	15– 54
TBGP-CGP, cells $\times 10^7$/kg	34.6 ± 15.6	12– 73

TABLE VI

Influence of epinephrine and exercise on the size of the TBGP and the CGP

Experimental condition	TBGP	CGP
	$\times 10^7$ *G/kg*	$\times 10^7$ *G/kg*
Control study*	68.2 (46–105)	34.2 (17–54)
After epinephrine infusion*	70.2 (45–117)	50.5 (31–70)
Control study†	48.2 (34– 56)	24.2 (16–41)
After exercise†	47.1 (36– 53)	38.6 (28–51)

* These values are the mean of studies on 8 subjects.
† These values are the mean of studies on 3 subjects.

Similar studies were carried out in three additional subjects except that a 440 yard run at the completion of the infusion was used to mobilize granulocytes. Under the influence of exercise the TBGP size was also unchanged and again the cells were shifted into the CGP to increase its size by 60 per cent (Table VI).

DISCUSSION

The results of these studies indicate that labeled granulocytes are distributed in a pool of cells which is larger than that calculated from the blood volume and from the concentration of granulocytes in the circulating blood. This dilution accounts, in part at least, for the previous observation (1), confirmed herein, that only about 50 per cent of the infused cells can be accounted for in the CGP at the completion of an infusion. An average of 79 per cent of the infused, labeled cells was present in the CGP following the administration of epinephrine or after physical exercise.

Since only 71 to 88 per cent of the injected cells was found in the CGP following epinephrine infusion (Table I) and 54 to 100 per cent after vigorous exercise (Table II), it may be argued that a variable proportion of the labeled cells was damaged irreversibly or that this proportion of the label eluted rapidly from the granulocytes. However, in view of our previous studies relevant to damage and elution (3), it seems unlikely that either damage or elution accounts for this immediate loss of cells from the circulation. A more reasonable explanation for our inability to account for all of the injected cells in every experiment is that epinephrine and exercise did not mobilize all of the granulocytes in each subject. Indeed, this is suggested by the considerable variation of 0 to 278 per cent in neutrophilic response from person to person.

Equilibration between the granulocytes in the CGP and in the "noncirculating pool" was both rapid and complete. When the "noncirculating granulocytes" were mobilized into the CGP within minutes by either epinephrine or exercise (Table III), there was no change in the specific activity of the cells in the CGP. Therefore, these two pools may be considered as one pool kinetically, and the size of this pool (TBGP) may be determined by the use of the isotope dilution principle.

Measurement of the TBGP in normal subjects revealed that the mean TBGP is 2.1 times the size of the CGP. Although direct quantitative measurements of the size of the total blood granulocyte pool have not been made before, it was estimated that there are as many granulocytes in the marginal pool as in the circulating blood (7).

It is interesting that neither epinephrine nor exercise influenced the size of the TBGP, although both the drug and the physical exertion caused a shift of cells from the noncirculating pool to the CGP (Table VI). This type of alteration in the distribution of granulocytes within the blood has been the subject of speculation for many years and was called "pseudo-leukocytosis" by earlier investigators (2, 8). The present study provides the first evidence for the existence of this type of leukocytosis. One might speculate that the acute neutrophilia which is associated with epileptiform seizures (9) and paroxysmal tachycardia (10) may also be of this type.

Our studies do not provide evidence for the anatomical location of the noncirculating pool of granulocytes. However, as indicated in the introduction, there is abundant evidence that there is a continuous exchange of granulocytes between the free-flowing axial blood and the more slowly moving marginal stream (2). Vejlens (2) showed that when the velocity of blood flow is reduced, granulocytes tend to assume a marginal position, become adherent to the walls of small veins and are withdrawn from the axial stream. When the velocity of blood flow is increased, granulocytes enter the axial stream and are car-

ried into the circulating blood. Since both exercise and epinephrine increase blood flow, it is reasonable to assume that these stimuli produce granulocytosis by mobilizing marginated cells. The work of Ambrus and Ambrus (11) and of Bierman and colleagues (12) suggests that the vascular system of the lungs is of particular significance in the margination of granulocytes. The importance of the vascular system of the splanchnic viscera (13) and extremities (14) has been demonstrated by the work of others. Apparently, the spleen plays a minor role in this regard, since granulocytosis induced by epinephrine is neither abolished nor diminished in splenectomized subjects (9).

SUMMARY

1. When granulocytes are labeled *in vitro* with radioactive diisopropylfluorophosphate and returned to the circulation of the donor, only about 50 per cent of the labeled granulocytes is present in the circulation at the completion of the infusion. It was postulated previously that the initial rapid egress of 50 per cent of the cells represented distribution of the injected granulocytes in a larger pool than that calculated from the blood volume and the venous granulocyte count. The experiments reported herein were designed to test the validity of this hypothesis. The following observations were made. a) An average of 78.8 per cent of the labeled granulocytes which were infused could be accounted for in the circulating blood when epinephrine was given with the infused cells. b) An average of 79 per cent of the labeled granulocytes was found in the circulation when the subjects exercised immediately following the completion of the infusion. c) Neither epinephrine nor exercise increased the size of the total distribution compartment (total blood granulocyte pool), although both the drug and physical exertion increased the size of the circulating granulocyte pool. d) Granulocytes damaged by storage could not be prevented from leaving the circulation by the administration of epinephrine. e) When granulocytes were mobilized into the circulation 3 hours after the infusion of labeled granulocytes, there was no change in the specific activity of the cells in the circulation.

2. These studies are interpreted as indicating that infused granulocytes are distributed in a total blood granulocyte pool (TBGP) made up of two compartments which are in rapid equilibrium with each other. These pools have been designated the circulating granulocyte pool (CGP) and the marginal granulocyte pool (MGP).

3. The size of these pools has been measured in 45 normal male subjects. The mean values $\pm$ 1 SD, expressed as number of cells $\times 10^7$ per kg of body weight, were as follows: TBGP, 65.3 ± 22.7; CGP, 30.7 ± 11.8; and MGP, 34.6 ± 15.6.

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OBSERVATIONS ON PHOSPHATE TRANSPORT IN EXPERIMENTAL RENAL DISEASE *

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In the normal adult, the renal excretion of phosphate may be varied over a wide range depending on the level of phosphate intake. When the diet contains the usual amounts of phosphate (about 1 g of phosphate per day), only a small fraction of the filtered phosphate is excreted in the urine. This fraction is decreased when the dietary phosphorus is low and increased when it is high. Homeostasis is thus maintained with only slight fluctuations of the plasma phosphate concentration (2–6).

In the course of advancing renal failure in man, normal plasma phosphate concentrations typically prevail until the glomerular filtration rate (GFR) falls below 25 to 30 ml per minute (7). This occurs by virtue of the fact that, as GFR decreases, the fraction of the filtered phosphate excreted increases proportionately; as a result, phosphate balance may be maintained in the presence of a decrease in the filtered load. However, when the GFR decreases below 25 ml per minute, the filtered load of phosphate is decreased to such an extent that even the excretion of a large fraction of the filtered load fails to maintain phosphate homeostasis, and hyperphosphatemia supervenes. The serum phosphate concentration will then increase until the filtered load of phosphate is sufficiently high for the re-establishment of phosphate balance. Advanced bilateral renal disease in man thus is characterized by hyperphosphatemia, but the degree of phosphate retention is minimized by the continuing excretion of a large fraction of the filtered phosphate.

The mechanism underlying the excretion of an increasing fraction of the filtered phosphate has been variously attributed to secondary hyperparathyroidism and to an inability of the diseased kidney to reabsorb phosphate (7, 8). Because renal insufficiency due to bilateral renal disease causes many alterations in the composition of extracellular fluid that could secondarily influence phosphate transport, it seemed desirable to study phosphate excretion by the diseased kidney under conditions in which the variables of extrarenal factors were minimized. The experimental model of permanent unilateral renal disease in dogs appeared to be well suited for such an investigation (9). This model makes possible the serial and simultaneous measurement of clearances in the diseased and normal kidneys of the same animal; owing to the presence of one intact kidney, the essentially normal composition of the extracellular fluid is maintained. Any consistent differences in the pattern of phosphate excretion between the diseased and contralateral control kidney might reflect alterations in phosphate transport induced by the renal disease per se.

METHODS

Thirty-four experiments were performed on 26 female mongrel dogs weighing 6 to 20 kg. In each animal, one of three types of unilateral renal disease was induced: *1*) aminonucleoside-induced nephritis, *2*) chronic pyelonephritis, *3*) antikidney serum glomerulonephritis. In one dog, bilateral pyelonephritis was induced.

Techniques for induction of these diseases, their histologic features, and details of the clearance measurements have been described in previous publications (9–11).

Patterns of phosphate excretion were studied by simultaneous determination of the clearances of phosphate and

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TABLE I

Patterns of phosphate excretion of diseased and intact kidneys under baseline conditions *

Clearance period	Plasma P	C_{Cr}		V		UV_P		C_P		$C_P/C_{Cr} \times 100$	
		Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
	μmoles/ml	*ml/min*		*ml/min*		*μmoles/min*		*ml/min*			
Dog A	Antikidney serum nephritis, wt, 7.8 kg										
1	1.52	7.1	27.0	1.08	3.75	2.23	7.98	1.47	5.26	21.0	19.5
2	1.61	6.5	23.9	0.89	2.96	2.61	8.78	1.62	5.45	24.8	22.8
3	1.61	6.8	26.3	0.86	2.93	3.19	11.2	1.98	6.97	28.9	26.5
Dog B	Pyelonephritis, wt, 8.6 kg										
1	1.24	4.66	50.4	0.578	3.61	0.841	12.0	0.675	9.63	14.6	19.1
2	1.31	3.16	45.7	1.000	2.97	0.590	12.2	0.450	9.27	14.2	20.3
3	1.17	3.96	45.7	1.09	2.44	0.738	11.6	0.629	9.85	15.9	21.6
4	1.25	4.42	49.4	1.13	3.13	0.860	14.8	0.687	11.8	15.6	24.0
5	1.24	3.72	42.4	1.09	2.81	0.777	12.5	0.624	10.0	16.7	23.6
6	1.48	4.99	54.1	0.708	4.24	1.28	16.1	0.865	10.9	17.2	20.1
7	1.53	4.22	55.1	0.659	4.44	0.916	16.6	0.600	10.8	14.2	19.6
8	1.40	4.35	52.9	0.745	4.72	1.07	15.8	0.769	11.3	17.7	21.4

* Dogs were maintained on an *ad lib.* diet of Purina dog chow. The clearance periods were usually 10 to 20 minutes in duration. P = phosphate, C_{Cr} = creatinine clearance, V = urine volume, UV_P = phosphate excretion rate, C_P = phosphate clearance.

creatinine in the diseased and contralateral control kidneys. Creatinine was measured by the method of Bonsnes and Taussky (12) and phosphate by the method of Gomori (13).

RESULTS

Baseline pattern. Eighteen experiments were performed in 16 fasting dogs without phosphate loading. Each experiment consisted of three to ten clearance periods.

Two representative experiments are shown in Table I. In Dog A, the fraction of filtered phosphate excreted (clearance ratio C_p/C_{Cr}) was closely comparable in the experimental and control kidneys despite a fourfold difference in filtration rates. Similar results were obtained in five experiments in five dogs with pyelonephritis and in five experiments in four dogs with antikidney serum nephritis.

TABLE II

Pyelonephritic dogs with moderate phosphate loading *

Clearance period	Plasma P	C_{Cr}		V		UV_P		C_P		$C_P/C_{Cr} \times 100$		Reab. P	
		Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
	μmoles/ml	*ml/min*		*ml/min*		*μmoles/min*		*ml/min*				*μmoles/min/ml GF*	
Dog A	Wt, 15.9 kg												
1	3.21	26.4	42.1	1.20	1.64	45.3	75.1	14.1	23.4	53.4	55.6	1.49	1.42
2	3.42	26.1	42.2	1.34	1.87	51.9	82.0	15.2	24.0	58.2	56.9	1.43	1.48
3	3.23	26.4	41.8	1.53	2.09	56.8	87.6	17.6	27.2	66.7	65.1	1.08	1.13
4	3.42	25.9	41.2	1.65	2.29	57.5	91.6	16.8	26.8	64.9	65.0	1.20	1.20
Dog B	Wt, 18.2 kg												
1	4.35	20.3	59.9	1.92	3.88	58.8	146.4	13.5	33.6	66.5	56.1	1.45	1.91
2	4.58	24.5	44.7	2.22	3.53	76.6	129.8	16.7	28.3	68.2	63.3	1.45	1.68
3	4.68	21.1	45.9	2.04	3.78	67.8	143.9	14.5	30.8	68.7	67.1	1.46	1.54
4	4.84	23.8	42.6	2.34	3.63	80.8	141.7	16.7	29.3	70.2	68.8	1.44	1.51
Dog C	Wt, 15.4 kg												
1	4.16	31.7	52.4	1.53	1.88	71.1	117.6	17.1	28.3	53.9	54.0	1.92	1.92
2	4.48	29.5	47.1	1.62	1.90	78.4	122.6	17.5	27.3	59.3	58.0	1.82	1.88
3	4.68	27.9	49.6	1.62	2.18	78.4	139.2	16.8	29.8	60.2	60.1	1.87	1.87
4	4.68	25.7	48.6	1.62	2.30	76.8	142.4	16.4	30.4	63.8	62.5	1.69	1.75

* Dogs received 4 g of ammonium chloride daily for 1 to 3 days before the study. Buffered phosphate (pH 7.4) was infused at a rate of 0.15 mmole phosphate/min. Creatinine was infused at a rate of 7 mg/min and PAH at a rate of 2.5 mg/min. Reab. P = reabsorbed phosphate, C_{Cr} = creatinine clearance, V = urine volume, UV_P = excreted phosphate, C_P = phosphate clearance.

The data on Dog B are representative of experiments in which the difference in clearance ratios was somewhat more marked. In the experiment depicted, the diseased kidney excreted a smaller fraction of its filtered phosphate than did the contralateral kidney. Comparable results were obtained in one other dog with pyelonephritis, in two experiments on a dog with aminonucleoside nephritis, and in two experiments on two dogs with antikidney serum nephritis. In only one experiment (a dog with pyelonephritis) was the clearance ratio higher on the diseased side. These small differences in clearance ratios were not related to *1*) the difference of filtration rates, *2*) the interval between induction of the experimental lesion and study, *3*) the serum phosphate concentration, and *4*) the absolute value of the clearance ratios. In any one experiment, the relationship between the clearance ratios was consistent in all clearance periods.

Phosphate loading. Thirteen experiments were performed in eight pyelonephritic dogs in which the serum phosphorus concentration was moderately elevated as a result of intravenous infusions of buffered phosphate; 1.5 to 4-fold differences in filtration rates were observed in this group of ani-

TABLE III

T_{mP} in unilateral renal disease *

Time	Plasma P	C_{Cr}		UV_P		Reab. P		T_{mP}		T_{mP}/C_{Cr}	
		Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
min	*μmoles/ml*	*ml/min*		*μmoles/min*		*μmoles/min*		*μmoles/min*		*μmoles/min/ml GF*	
Dog A	Pyelonephritis, wt, 9.5 kg										
0–20	0.910	13.8	27.2	2.00	3.65	10.6	21.0				
20–36	0.906	11.7	28.0	1.57	2.99	9.0	22.4				
36–54	0.910	9.1	24.8	0.963	1.93	7.32	20.7				
55	Infusion of 0.16 mmole phosphate/min begun										
54–86.5	3.02	12.8	26.9	9.26	2.68	29.4	78.5				
86.5–100	3.60	11.5	27.0	20.5	58.2	21.0	39.0				
100–117.5	4.21	12.6	28.2	34.5	70.2	18.5	48.5				
117.5–138	4.74	11.1	29.7	35.3	92.2	17.3	48.6				
139	Infusion of 0.33 mmole phosphate/min begun										
138–171.5	7.04	13.6	28.0	64.1	145	31.6	52.0				
171.5–187.5	7.67	16.0	30.5	89.1	191	33.9	43.0				
187.5–204	8.20	16.0	33.6	102	216	29.0	60.0	31.5	51.7	2.06	1.68
Dog A′	Same dog studied 10 days later										
0–10	1.05	9.97	23.8	2.39	5.40	8.11	19.5				
11.5	Infusion of 0.16 mmole phosphate/min begun										
10–32.5	2.38	8.88	19.9	8.02	19.8	13.1	27.6				
32.5–46	3.02	9.29	20.7	14.7	31.8	13.3	30.8				
46–58.5	3.74	8.71	11.4	18.3	23.8	14.3	18.8				
60	Infusion of 0.33 mmole phosphate/min begun										
58.5–81	6.22	9.15	21.8	36.0	87.4	21.1	48.4				
81–98	7.58	10.72	22.5	59.5	122	21.8	49.0				
100	Infusion of 0.82 mmole phosphate/min begun										
98–129	13.7	9.84	23.7	109	264	26.0	62.0				
129–141	16.3	10.77	24.2	161	347	14.0	47.0	20.7	51.6	2.05	2.24
Dog B	Antikidney serum, wt, 12.3 kg										
0–21	1.37	1.55	53.2	0.259	9.21	1.86	63.5				
21–43.5	1.34	1.70	52.8	0.103	8.73	2.18	62.3				
50	Infusion of 0.16 mmole phosphate/min begun										
43.5–100	3.42	2.24	59.3	4.39	107	3.25	96.0				
100–115	3.71	2.38	53.5	7.42	129	1.42	69.0				
116.5	Infusion of 0.33 mmole phosphate/min begun										
115–138.5	5.00	2.20	59.0	7.58	193	3.42	102				
138.5–149	5.84	2.42	59.0	10.3	237	3.80	107				
149–158.5	6.32	2.78	60.4	13.8	275	3.80	107				
160	Infusion of 0.82 mmole phosphate/min begun										
158.5–180	10.1	2.81	53.9	22.4	451	6.00	93				
180–185	11.4	2.97	53.0	28.0	511	5.50	91	4.5	98	1.70	1.71

* Continuous infusions of creatinine (7 mg/min) and para-aminohippuric acid (2.5 mg/min) were maintained in all studies. P = phosphate, C_{Cr} = creatinine clearance, UV_P = phosphate excretion rate.

mals. The pattern of phosphate excretion for three representative experiments is shown in Table II. The fractions of filtered phosphate excreted by diseased and control kidneys were in close agreement. There was no statistically significant difference ($p > 0.05$) in any one experiment or in group data when the results of all experiments were pooled (degrees of freedom = number of clearance periods minus 1 in a paired *t* test). In some experiments, the serum phosphate concentration was sufficiently high that saturation of the tubular capacity for phosphate reabsorption (Tm_P) might have occurred. For this reason, the values for net phosphate reabsorption per unit of GFR are also shown in Table II.

The plasma phosphate concentrations were elevated progressively to values well in excess of Tm_P in three experiments on two dogs (Table III). The value for Tm_P was calculated as the mean of the observed rate of phosphate reabsorption (micromoles per minute) of all clearance periods in which the plasma phosphate concentration was 5 μmoles per ml or higher (3). Although the absolute value of the Tm_P (micromoles per minute) was always lower on the experimental side, the ratio Tm_P/GFR was closely comparable in the experimental and control kidneys. Furthermore, the differences of these ratios between kidneys were not consistent in direction; in the dog with pyelonephritis, the Tm_P/GFR ratio was higher in the experimental kidney in one study (A, Table III) and higher in the control kidney in a subsequent study (A', Table III). In Dog B, the ratio was nearly identical in the two kidneys. The comparability of the Tm_P/GFR in the two kidneys is particularly noteworthy in the latter dog, since filtration rates differed by a factor of 20.

DISCUSSION

In most of these experiments, the pattern of phosphate excretion was comparable in the diseased and contralateral control kidneys, regardless of the type of experimental renal disease studied or of the severity of the renal lesion. The similarity of the pattern in the two kidneys was particularly impressive in experiments with phosphate loading. Total excretion of phosphate and phosphate clearances were always reduced in the diseased organ, but this reduction was proportional to the decrease of the GFR. Intrinsic failure of tubules to perform phosphate transport was not evident.

In several of the experiments performed without phosphate loading, a difference of moderate degree was observed in the pattern of phosphate excretion between diseased and normal kidneys. In these experiments the diseased kidney generally excreted a smaller fraction of the filtered phosphate than did the control organ. The explanation for this phenomenon is not apparent; however, if nephrons in the diseased kidney lost their capacity for phosphate reabsorption, the differences between the two kidneys would have been in the opposite direction.

The results of the present studies may be interpreted in terms of current concepts of renal phosphate transport. Serum inorganic phosphate is filtered at the glomerulus and reabsorbed by the tubules to a variable extent. A tubular maximum for phosphate transport can be demonstrated when the serum phosphate concentration is progressively increased. However, in contrast to the Tm for glucose, the Tm_P is highly variable, in both man and dog (3, 14). Thus at high filtered loads, the absolute rate of phosphate reabsorption often varies appreciably from period to period in individual studies, and the calculated value for Tm_P may vary over a wide range when repeated studies are performed on the same subject. The reasons for this variability are not completely clear, but two factors appear to be of major importance. *1*) Parathyroid hormone is thought to decrease the maximal rate of net phosphate reabsorption, and the measurement of theTm_P may require the induction of changes in parathyroid function. These changes may be brought about by hyperphosphatemia per se or by hyperphosphatemia-induced depression of the serum calcium concentration. *2*) Phosphate may be secreted by the tubules as well as reabsorbed. Phosphate secretion is well established in some species but has not been unequivocally established in mammals (15, 16). It is possible, in the experiments in which the clearance ratio was lower in the diseased kidney, that less phosphate secretion was accomplished by the tubules of the diseased kidney.

Two experimental details require special comment. The variation of Tm_P noted in these experiments was of the same order of magnitude as

that observed by most other investigators (3, 14). In the experiments with moderate phosphate loading (Table II), the animals were acutely acidotic. It has been shown previously that acidosis does not influence the Tm_P (3).

If experimental renal disease in the dog is functionally comparable with spontaneous renal diseases in man, the characteristically high clearance ratios of advanced bilateral human disease require explanation. Inability of the surviving tubules to transport phosphate effectively would appear to be unlikely on the basis of the dog experiment. Bilateral renal disease in the dog with moderate uremia is characterized by patterns of phosphate excretion very much like those of bilateral human disease. This is illustrated in Table IV, in which several experiments on a dog with bilateral pyelonephritis are shown. The combined GFR (i.e., two kidneys) was 11 to 13 ml per minute and the plasma urea nitrogen concentration was approximately 60 mg per 100 ml. From 37 to 59 per cent of the filtered phosphate was excreted (a range considerably higher than that noted in animals with unilateral renal disease). Despite the marked lowering of GFR and a normal dietary intake, hyperphosphatemia was prevented.

The excretion of an increasing fraction of filtered phosphate in bilateral chronically diseased kidney may therefore represent an adaptive change in phosphate transport which facilitates the maintenance of phosphate balance. Of the various factors responsible for the modifications in phosphate transport, small changes of the plasma phosphate concentration and secondary hyperparathyroidism must be considered. If total phosphate reabsorption is decreased in proportion to the reduction of the GFR, as the present data indicate, even a slight increase of the plasma phosphate concentration, at a given level of GFR and phosphate reabsorption, would result in an increase of the clearance ratio. In the clinical situation, the change in plasma phosphate concentration may be so subtle as to escape detection, and the plasma phosphate concentration may "remain within normal limits" while the clearance ratio is increased. That minimal hyperphosphatemia is probably not the sole cause of the increased clearance ratio in bilateral human disease is suggested by the observation that the clearance ratio remains high after the serum phopshate concentration has been reduced by a low phosphate diet Basagel administration (17). In addition, preliminary experiments have shown that the very high clearance ratio in man with advanced renal insufficiency can be substantially lowered by an infusion of calcium salts, a procedure that probably decreases parathyroid hormone release (18).

TABLE IV

Bilateral pyelonephritis *

Date	Serum P	C_{Cr}	C_P	$C_P/C_{Cr} \times 100$
	μmoles/ml	*ml/min*	*ml/min*	
5/6	1.42	11.1	4.11	37.0
5/20	1.63	11.6	5.31	45.8
5/24	1.70	12.6	5.58	44.3
6/3	1.74	11.2	6.58	58.8

* Each value is the mean of at least 3 clearance periods. P = phosphate, C_{Cr} = creatinine clearance, C_P = phosphate clearance.

SUMMARY

1. Phosphate transport was studied in dogs with unilateral renal disease. The experimental model made possible simultaneous clearance measurements in diseased and contralateral control kidneys in the absence of uremic changes in the extracellular fluid.

2. Under these conditions, phosphate transport, as measured by Tm and by moderate phosphate loading, was entirely comparable in the diseased and normal organs when transport was expressed per unit of glomerular filtrate. In the absence of phosphate loading, the fraction of filtered phosphate excreted by the diseased kidney generally approximated that of the contralateral control kidney.

3. It is concluded that the intrinsic capacity of the residual nephrons in the dog to transport phosphate remains essentially intact, and it is suggested that the excretion of an increasing fraction of filtered phosphate in advancing renal disease in man may relate principally to adaptive changes induced by the uremic state.

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THE EFFECT OF 2-DEOXY-D-GLUCOSE INFUSIONS ON LIPID AND CARBOHYDRATE METABOLISM IN MAN*

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At the present time, the direction of change of the plasma free fatty acid (FFA) level is considered to serve as an index to the pattern of fat metabolism, representing an indicator of the balance between fat storage and fat mobilization. In the fasting, nonexercising individual, a rising plasma FFA level suggests net fat mobilization and a falling FFA level suggests net fat storage. If the status of carbohydrate metabolism does relate to fat metabolism, then plasma FFA levels may be expected to change in a number of experimental situations. Dole was the first to demonstrate that glucose and insulin administration decreased fasting FFA levels (1) and Bierman, Dole and Roberts that patients with diabetes mellitus have high FFA levels (2). Experimental inteference with carbohydrate metabolism might also be expected to influence plasma FFA levels. The availability of 2-deoxy-D-glucose (2-DG) has made it possible to test this hypothesis. 2-DG is phosphorylated to 2-deoxyglucose-6-phosphate (2-DG-6-P), and it has been postulated that the 2-DG-6-P may compete for transport into the cell with glucose-6-phosphate (3). The purpose of this study was to observe the effect of 2-DG administration on FFA levels in normal human subjects and to study in addition the effect of glucose, insulin, fructose and lactate upon the pattern of response to 2-DG.

MATERIALS AND METHODS

2-DG was purchased in the chemically pure form from the Nutritional Biochemicals Corporation. The compound was dissolved in distilled water, passed through a Seitz filter, and stored in a sterile container for intravenous administration. Aliquot samples were tested for bacterial contamination. 2-DG was prepared in 20 per cent solution and stored in the cold. For intravenous administration the final volume was made up to 100 ml with normal saline.

Plasma glucose was measured by the Nelson modification of the Somogyi method (4), which also determines blood 2-DG levels. 2-DG levels were not specifically determined in these experiments but have been previously reported following the administration of a comparable amount of compound to humans. The 2-DG plasma levels rarely exceed 10 mg per 100 ml (5). FFA was measured by the method of Dole (1) as modified by Trout, Estes and Friedberg (6). Plasma lactate was measured by the Barker-Summerson method (7). Urinary epinephrine and norepinephrine levels were measured by the bioassay technique of von Euler (8).

Eleven subjects were selected for these studies on the basis of normal body build and no evidence of metabolic or endocrine abnormality. The subjects were either medical students or hospital patients with minor illnesses. All subjects were studied in the morning after a 12 hour overnight fast. On entering the laboratory, they were placed in bed and remained recumbent and quiet for the duration of the study. Needles were placed in a vein of each arm, one for the intravenous infusion of 2-DG, the other for sampling. Each sample consisted of 10 ml of blood drawn into heparinized syringes and immediately placed on ice prior to centrifugation in the cold. Two or three pre-infusion samples were drawn starting at 10 minutes after placement of the needles. The 2-DG was infused at a constant rate over a 30 minute period. The total dose was 60 mg per kg in all but two studies (one with 50 mg per kg and one with 70 mg per kg). Blood samples were then drawn at 5 to 15-minute intervals during the first hour of the experiment and at 30-minute intervals after that period. The experiments lasted approximately 3 to 4 hours. Signs and symptoms of drug effect were recorded in each instance. These included sweating, somnolence, hunger, and occasionally, nausea and restlessness. In no instance were the symptoms sufficiently disturbing to the subject to warrant discontinuing the study.

In the studies of sodium lactate, this compound was infused intravenously at various dose levels before, during or after the attainment of a clinical 2-DG effect. In other experiments, 25 or 50 g of glucose and 22 g of fructose were given intravenously after a 2-DG effect was apparent. Hyperglycemic-free insulin (HGF) was also studied in doses up to 8 U, administered intravenously

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in 2 to 3 minutes, and also after a 2-DG effect had occurred.

RESULTS

Figure 1 depicts a representative response to the infusion of 2-DG. The increase in plasma FFA began shortly after the start of the infusion, rose rapidly to a peak, and gradually fell toward the control levels over a 4 hour period. Plasma glucose levels first began to rise concurrently with the initial rise in FFA level but were more gradual in reaching a peak. Part of the immediate initial rise may be contributed by the 2-DG itself. In only one subject did plasma glucose fall toward pre-infusion levels within the 3 hour period of study. Blood lactate levels also rose gradually over a period of hours. Table I summarizes the nine experiments of unmodified 2-DG administration to normal subjects. It can be noted that irrespective of the pre-infusion FFA level, the response to 2-DG administration was invariably a rise ranging from 1.5 (Subject C.C.) to 10 times (Subject J.S.) the pre-infusion level. There was overlap of only one control (C.C.) and one maxi-

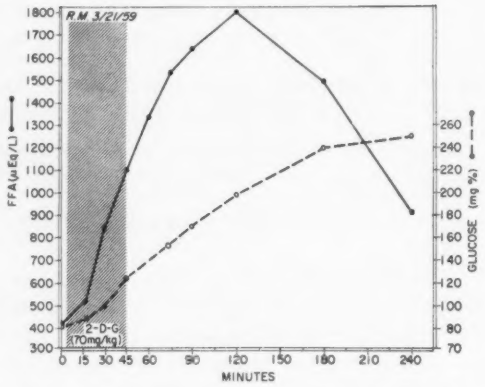

FIG. 1. A REPRESENTATIVE PATTERN OF RESPONSE OF PLASMA FREE FATTY ACID AND GLUCOSE LEVELS FOLLOWING AN INFUSION OF 2-DEOXY-D-GLUCOSE.

TABLE I

Plasma free fatty acid, glucose and lactate levels before and after intravenous infusion of 2-deoxy-D-glucose (2-DG)

Subject	Control	Minutes after completion of infusion 5–10	15–20	30	45	60	90	120	150	180	210	
Free fatty acid (μEq/L)												
1. G.S.	587	910	1,402	1,591	1,424	1,059	1,024	1,156	1,202	998		
2. J.V.	324	644	1,039	1,279	1,267		1,178	1,094	1,025	1,016		
3. R.M.	416	1,100	1,332	1,532	1,638		1,798	1,494		914		
4. C.C.	846		1,295	1,257		1,193	1,116		1,168		1,011	
5. M.A.	442	1,046		1,221	954	948	1,024	856				
6. L.G.	893	1,841		2,200	2,387	2,396	2,299	2,305	2,016		1,048	
7. R.D.	539	1,209	1,812	2,091	1,810		1,601	1,125				
8. R.Me.	356	425	796		767	875	719					
9. J.S.	82		485	649	769	822	731	748				
Glucose (mg/100 ml)												Glucose release rate (g/hr)
1. G.S.	90	107	116	124	142	151	146	148	132	120		27.6
3. R.M.	83	124		156	170		198	240		250		26.7
4. C.C.	119		133	142		154	154		161			8.7
5. M.A.	87	105		121	143	154	165	180				24.0
6. L.G.	101	131		156	165	194	219	233				51.6
7. R.D.	89	121	151	189	215		233	233				91.8
8. R.Me.	95	109	135		154	160	170					51.9
9. J.S.	85		108	135	151	158	183	189				39.0
											Mean	40.2
Lactate (mg/100 ml)												
3. R.M.	7	7		9	7		10	12		11		
4. C.C.	14		15	20		24	34		38			
5. M.A.	12	12			24		35		44			
10. H.J.*	10		9		11							
11. A.N.*	11		18	31		33						

* Subjects received insulin at 60 minutes and are not included in control data of FFA and glucose tables.

mal response value (J.S.), representing a statistical probability of difference at the $p < 0.001$ level. The average time to obtain a maximal response as measured from the completion of the infusion was 45 minutes with a range of 20 to 75 minutes. FFA level elevations persisted for the duration of each experiment.

A significant rise in plasma glucose levels also occurred in every instance. The maximal rise in glucose was frequently not attained within the duration of these studies but, when reached, rose to an average of 196 mg per 100 ml. In all of the studies in which both FFA and glucose were measured, the first significant rise in glucose was well correlated temporally with the first significant rise in FFA level. As in Figure 1, the pattern of response of glucose levels shortly after the infusion was begun was linear and steep. During the period of linear rise of plasma glucose it is possible to make an approximate calculation of the rate at which glucose is being added to the total body water by way of glycogenolytic and gluconeogenetic processes. This is based on the previous finding that during 2-DG infusion glucose utilization is negligible (5) and that any rise in plasma glucose must be primarily due to glucose production by way of glycogenolysis and gluconeogenesis. In the subject depicted in Figure 1, during a half-hour period of nearly linear glucose rise (0–30 minutes after completing the infusion), the plasma glucose concentration changed 32 mg per 100 ml. For a calculated average total body water compartment of 60 per cent of body weight, this plasma concentration increase represents the addition of 26.7 g of glucose per hour. Table I lists the estimated rates of glucose release for the control subjects (mean, 40.2 g per hour). Any glucose utilization that occurred during these experimental periods and any renal excretory loss of glucose would serve to make the observed values less than the actual glucose production. Comparable calculations for FFA metabolism cannot be made, since the effects of 2-DG on specific reactions affecting FFA utilization and mobilization are not known.

Lactate. Table I lists the serum lactate levels in five subjects. In all subjects there was a rise which progressed throughout the study.

Effect of insulin on the response to 2-DG infusion. Figure 2 illustrates an experiment in which

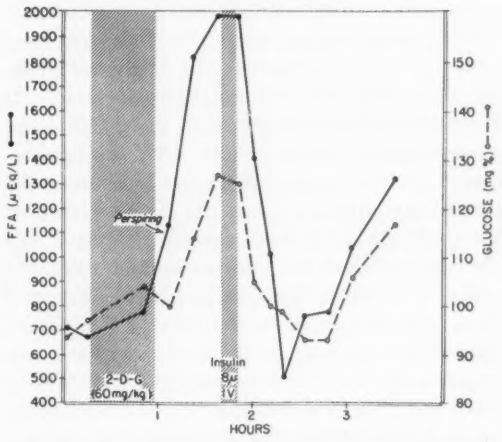

FIG. 2. THE EFFECT OF HGF INSULIN (0.1 U PER KG), INTRAVENOUSLY ADMINISTERED, UPON THE INCREASED PLASMA FREE FATTY ACID AND GLUCOSE LEVELS FOLLOWING THE INFUSION OF 2-DEOXY-D-GLUCOSE.

a dose of 8 U of HGF-free insulin was administered intravenously over a 3 minute period at a time when the FFA level was expected to be at a maximum. It will be noted that in this experiment, at the time insulin was administered, glucose and FFA levels were on the ascent. Shortly after insulin administration there was a rapid fall in FFA and glucose levels. This fall began to level out approximately 60 minutes after insulin administration. The fall in the FFA level was very sharp, decreasing from 1,993 to 505 μEq per L during a 25 minute period. Despite the rapid reversal in FFA and glucose levels, the subject continued to experience symptoms. At a later point in the study (not recorded in the figure), 25 g of glucose administered intravenously was accompanied by prompt cessation of the symptoms. A similar study of insulin effect in a second subject revealed the same pattern of response.

Effect of glucose, fructose and lactate on the response to 2-DG. Figure 3 illustrates a series of experiments in Subject G.S. studied on three different occasions. In addition to an unmodified 2-DG infusion study, noted as control, the effects of glucose and fructose on the 2-DG infusion were observed on other days. The peaks of the FFA levels were not the same for all three days but were of a comparable order of magnitude. The major difference between these studies is the rate of fall of the FFA level. After the glucose infu-

sion, it was considerably increased, control levels of FFA being reached 60 minutes following completion of the 2-DG infusion. In the control study, the FFA level was still markedly elevated 190 minutes following completion of the 2-DG. The reversal effect of glucose on the FFA level gradually diminished as plasma glucose levels returned toward pre-infusion values. At the completion of the study, the FFA level was again elevated. In the study in which fructose was infused, there was a slightly more rapid decline in plasma FFA level as compared with the control day. The plasma glucose level during the fructose experiment was higher than that of the control, a reflection of fructose conversion to glucose which may account for the partial reversibility seen in this experiment.

Five hundred ml of ⅙ M sodium lactate infused simultaneously with 2-DG did not alter the rate of FFA decline. Several other series of experiments using infusions of sodium lactate at varying time intervals after 2-DG administration also seemed to be ineffective in reversing FFA level response to 2-DG.

Catechol amine excretion. In four subjects, urinary catechol amine levels were determined before, during and after 2-DG infusion (Table II). There was a striking increase in epinephrine excretion in all studies. Because of the proportionality method involved in the bioassay procedure, the norepinephrine levels could not be accurately assessed in the presence of such large amounts of epinephrine. Accordingly, in two experiments norepinephrine levels were not calculated. In the other two experiments the levels tended to fall.

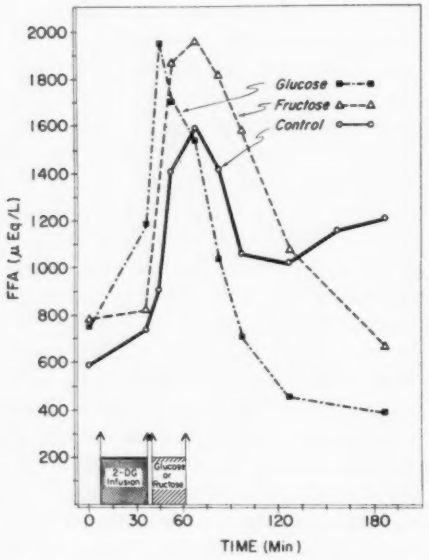

FIG. 3. THE EFFECT OF GLUCOSE AND FRUCTOSE UPON THE PLASMA FREE FATTY ACID AND GLUCOSE ELEVATIONS FOLLOWING INFUSION OF 2-DEOXY-D-GLUCOSE.

TABLE II

Catechol amine excretion rates before, during and after intravenous infusion of 2-deoxy-D-glucose

Subject	Period*	Epinephrine	Norepinephrine
		μg/hr	*μg/hr*
7. R.D.	I	1.55	6.01
	II	31.47	
	III	21.92	
8. R.Me.	I	4.78	34.65
	II	28.34	
	III	19.88	
9. J.S.	I	1.37	5.85
	II	6.79	3.35
	III	11.11	
11. A.N.	I	2.99	18.30
	II	10.57	2.31
	III	13.03	14.12

* Periods: I = before infusion; II = during infusion and 1 hour after infusion completed; III = 2 to 3 hours after infusion completed.

DISCUSSION

These studies are considered to provide further evidence for the thesis that the status of carbohydrate metabolism is an important determinant of the equilibrium of fat metabolism. Following 2-DG infusion, the sharp rise in FFA levels that occurred in the fasted, resting individual suggests a net increase in fat mobilization as compared with fat synthesis and utilization. The reversal of the 2-DG response by insulin, glucose and fructose would indicate that the effect of 2-DG upon lipid dynamics is most likely a result of the known action of 2-DG as a competitive inhibitor of glucose utilization. The actual mediating mechanisms of this glucose-lipid interrelationship are not specifically delineated by the experiments reported here. White and Engel have studied the effect of 2-DG upon adipose tissue *in vitro* and have not noted any alteration in FFA release into or uptake from

the media in such experiments (9). Data on the utilization of FFA by specific tissues samples *in vitro* in the presence of 2-DG have not been reported. Studies in this laboratory using C^{14}-labeled palmitate do not indicate any effect of 2-DG on the rate of disappearance of infused FFA in human subjects (10).

The FFA level rise observed in these studies might represent a response to 2-DG infusion which is mediated by lipid mobilizing processes present only in the intact subject. The symptoms of drowsiness, hunger and marked sweating suggest the response recognized as the one attending hypoglycemia. In the presence of 2-DG, although the measured plasma level of glucose is high, if cellular utilization of glucose cannot occur the organism can be considered to be functionally hypoglycemic. The relative unavailability of glucose to the central nervous system might then initiate the usual response of the organism to hypoglycemia which characteristically includes adrenal medullary secretion. Epinephrine and norepinephrine are both known to raise plasma FFA levels and to have an *in vitro* lipolytic property (1, 11, 12). In the studies reported here, the marked increases in urinary epinephrine excretion and the immediate rise in plasma glucose level that follows the 2-DG infusion would be consistent with the hypothesis that the FFA level rises are mediated by a mechanism involving the adrenal medulla.

Significant rises in plasma glucose invariably follow 2-DG infusion and sometimes persist for 6 to 8 hours. In this experimental situation, when glucose utilization is markedly blocked, the amount of net glucose added to the body water may be calculated. This net increment of glucose most likely represents both glucose release by way of glycogenolysis and glucose elaboration by the process of gluconeogenesis. Values of 9 to 90 g of glucose per hour were obtained by such calculations. The magnitude of these figures approximates, and in some instances exceeds, those estimates of Bearn, Billing and Sherlock (13, 14) and of Werk and associates (15).

The symptoms experienced as a result of the 2-DG infusion were reversed by the administration of glucose but not by insulin, although the FFA rise was equally well reversed by both procedures. The protective effect of glucose on 2-DG toxicity, as well as the failure of insulin to protect against 2-DG toxicity, has also been observed in mice (16). The lack of reversal by insulin may be due to the reduction of serum glucose subsequent to insulin injection. In this experimental situation the FFA level is being modified by an insulin-sensitive tissue (adipose), but the 2-DG effects on the central nervous system are occurring in an insulin-insensitive tissue (the brain).

Finally, with the thesis in mind that the key role of glucose metabolism in governing FFA levels might be concerned with glucose serving as a source of glycerol for the esterification of free fatty acids, lactate infusions were given so as to provide an alternate source of three carbon compounds. In our study of a series of lactate infusions at varying dosage levels and at different times with relation to 2-DG administration, we were unable to demonstrate any reversal of the plasma FFA response to 2-DG. However, Mueller who independently found that 2-DG infusions in dogs caused consistent plasma FFA rises, noted that the administration of lactate reversed the FFA rise (17). Our failure to demonstrate a similar lactate reversal effect in man may be due to the different dosages employed, or else to the species difference in the handling of lactate and the degree to which exogenous lactate is available to esterify fatty acids.

SUMMARY

1. 2-Deoxy-D-glucose (2-DG), a competitive inhibitor of glucose metabolism, was administered intravenously to a series of fasting, rested subjects.

2. Plasma levels of free fatty acid (FFA), glucose and lactate increased markedly following the infusion of 2-DG.

3. Insulin, glucose and fructose reversed the rise in plasma FFA but did not alter plasma lactate levels. Lactate infusion did not alter the FFA or glucose response to 2-DG. Insulin also reversed the glucose rise following 2-DG but not the clinical side effects.

4. Marked increases in urinary excretion of epinephrine accompany and follow the infusion of 2-DG and suggest that the mechanism mediating the plasma FFA response may involve the adrenal medulla.

5. 2-DG infusion in the fasting individual may provide a technique for making a quantitative es-

timate of the magnitude of net glucose release in man.

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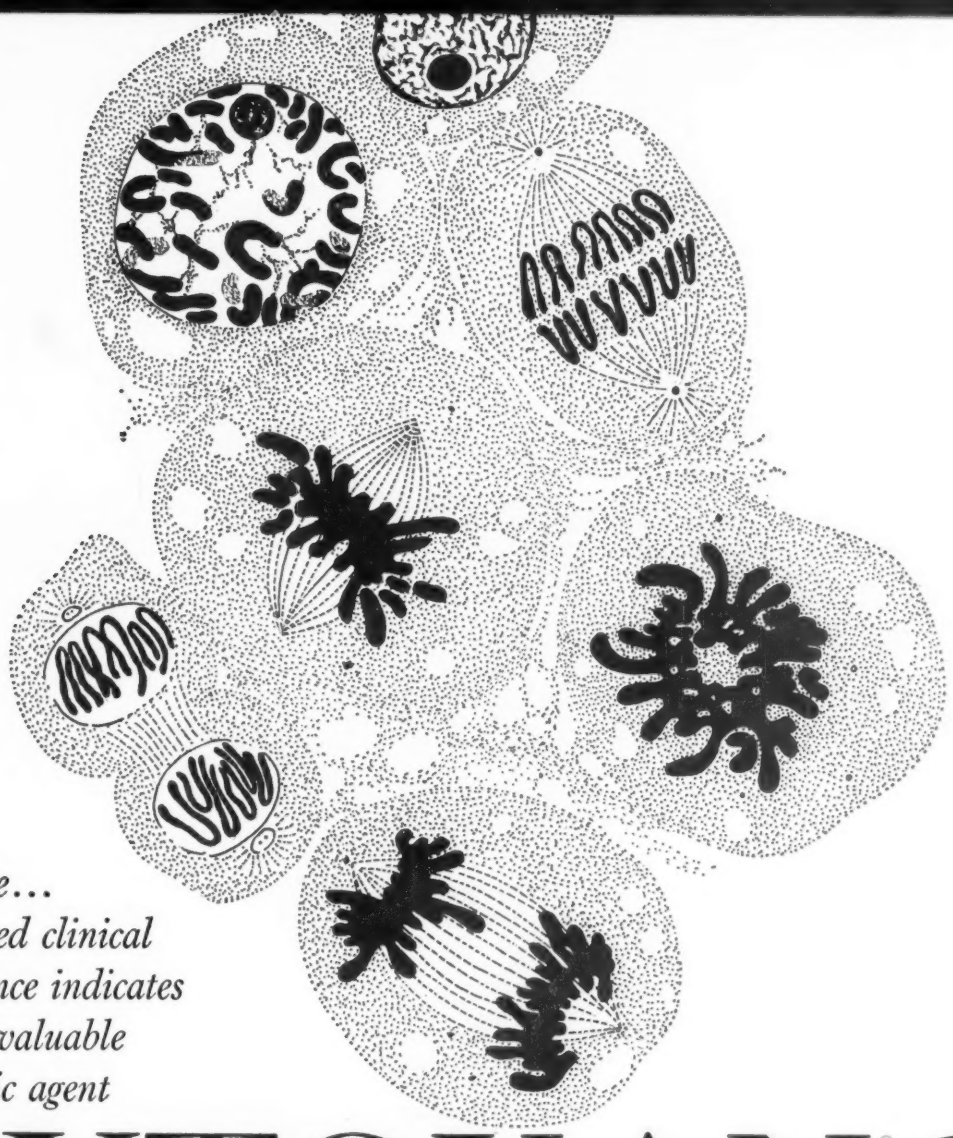

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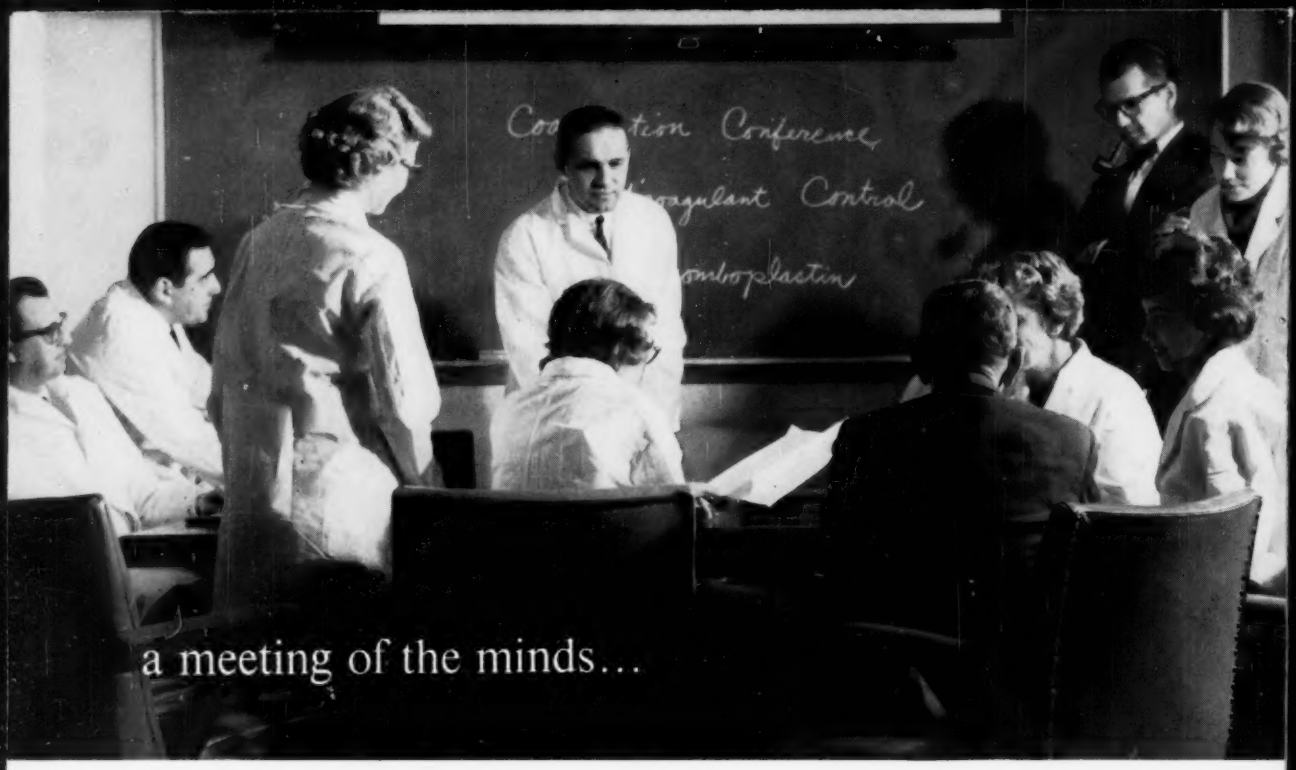

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